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DOCTORAL THESIS

Cyclophosphamide and ifosfamide: mechanisms of cytotoxic action and consequences for normal bladder function

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PhD Thesis

by

Kylie Alexandra Mills

Cyclophosphamide and ifosfamide:
mechanisms of cytotoxic action and
consequences for normal bladder
function

Submitted in total fulfilment
of the requirements of the degree
of Doctor of Philosophy by Research

March 2015

Abstract

This thesis investigates the urotoxic effects of the commonly used cytotoxic drugs cyclophosphamide and ifosfamide. Both drugs are well recognised for causing haemorrhagic cystitis and lasting adverse effects in the bladder including pain, increased urinary frequency and urgency and sensations of incomplete bladder emptying. These adverse effects have been largely attributed to the formation of the toxic metabolite acrolein which is excreted in the urine. However, another urinary metabolite of these drugs is chloroacetaldehyde and its role in urotoxicity has not been explored. Understanding more about what effects these drugs and their metabolites have on the bladder and its function may uncover possible targets for preventing, alleviating or treating the adverse urological effects and could lead to better drug toleration and better treatment outcomes.

The effects of acrolein and chloroacetaldehyde were investigated using cultured human urothelial cells as well as full thickness porcine bladder sections. Systemic administration of cyclophosphamide and ifosfamide was also performed in mice to study the effects of the endogenously produced metabolites on whole bladder and nerve function. A combination of pharmacological agents and chemical, mechanical and electrical stimuli were used to investigate and compare the responses of control and treated tissues.

Experiments measuring mediator release from urothelial cells implicated both acrolein and chloroacetaldehyde in the urotoxicity of cyclophosphamide and ifosfamide as both metabolites caused increased excitatory transmitter release from the cells. It was thought that the increase in excitatory transmitter release may contribute to bladder hyperactivity by activating and/or sensitising afferent nerves. Both metabolites also caused urothelial damage when applied to the luminal side of porcine bladder sections. However, despite loss of urothelial cells, the mediator release was comparable to controls suggesting enhanced release from each cell compensated for overall cell loss. Total afferent nerve activity was found to be increased in mice after treatment with either cyclophosphamide or ifosfamide due to enhanced activity of the low threshold nerve fibres. However, the heightened afferent activity observed in mice was not associated with increased excitatory urothelial mediator release or altered detrusor tone. This suggests that cyclophosphamide or ifosfamide treatment is able to enhance nerve activity via a mechanism independent of bladder function and that the bladder pain and urinary hyperactivity experienced by patients is primarily due to sensitisation of the afferent pathways.

Declaration

This thesis is submitted to Bond University in fulfilment of the requirements of the degree of Doctor of Philosophy by Research

This research represents my own original work towards this research degree and contains no material which has been previously submitted for a degree or diploma at this university or any other institution, except where due acknowledgment has been made.

Kylie Mills

2 March 2015

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Publications

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Mills, K. Grundy, L. Rose'Meyer, R. McDermott, C. Chess-Williams, R. (2014) Cyclophosphamide and ifosfamide enhancement of bladder sensory nerve activity. Clinical Oncology Society of Australia, Melbourne 2014. Published in Asia-Pacific Journal of Clinical Oncology, Special Issue: COSA's 41st Annual Scientific Meeting v.10 S8 pp134 (No. 226). Available online at: <http://onlinelibrary.wiley.com/doi/10.1111/ajco.12305/epdf>

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Kylie A Mills, Russ Chess-Williams, Catherine McDermott. (2013) The protective effect of N-acetylcysteine and Vitamin C on acrolein toxicity in human urothelial cells. 5th National Symposium on Advances in Gastrointestinal & Urogenital Research, Melbourne 2013

Chess-Williams R, Mills K, McDermott C, (2012) Acrolein, a metabolite of cyclophosphamide enhances basal ATP release and reactive oxygen species formation in cultured human urothelial cells. Proc. 42nd Annual Meeting of the International Continence Society, Beijing 2012. Available online at: <http://www.ics.org/2012/programme/search>

Kylie A Mills, Catherine McDermott, Russ Chess-Williams (2012) Effects of acrolein, a metabolite of Cyclophosphamide and Ifosfamide, on cultured human urothelial cells. Proc. ASCEPT Annual Meeting, Sydney 2012. Available online at: <http://www.ascept-apsa.com/wp-content/uploads/2012/07/ASCEPT-APSA-Poster-Abstracts-570-644.pdf>

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Abbreviations

ACh: acetylcholine	K ⁺ : potassium
AChE: acetylcholinesterase	KCl: potassium chloride
ADP: adenosine 5'-diphosphate	LDH: lactate dehydrogenase
AP-1: activator protein-1	LP: lamina Propria
AR: adrenoceptor	NA: noradrenaline
ASIC: acid sensing ion channel	Na ⁺ : sodium
ATP: adenosine 5'-triphosphate	NAC: N-acetylcysteine
Ca ²⁺ : calcium	NAD: nicotinamide-adenine dinucleotide
CAA: chloroacetaldehyde	NANC: non-adrenergic, non-cholinergic
cAMP: 3'-5'-cyclic adenosine monophosphate	NF-κB: nuclear factor-kappaB
cGMP: 3',5'-cyclic guanosine monophosphate	NGF: nerve growth factor
CGRP: calcitonin gene-related peptide	NO: nitric oxide
COX: cyclooxygenase	NOS: nitric oxide synthase
CPO: cyclophosphamide	ONOO ⁻ : peroxynitrite
CYP: cytochrome P450	p.o.: per oral
DAG: diacylglycerol	PACAP: pituitary adenylate cyclase activating peptide
Deg: degenerin Na ⁺ channels	PAG: periaqueductal gray
DRG: dorsal root ganglia	PARP: poly ADP-ribose polymerase
EFS: electrical field stimulation	PBS: painful bladder syndrome
EGF: epidermal growth factor	PGE ₂ : prostaglandin E2
EGFR: epidermal growth factor receptor	PLC: phospholipase C
ENaC: epithelial sodium channel	PMC: pontine micturation centre
EUS: external urethral sphincter	RNS: reactive nitrogen species
GAG: glycosaminoglycans	ROS: reactive oxygen species
GSH: glutathione	RT-PCR: reverse transcription polymerase chain reaction
H ₂ O ₂ : hydrogen peroxide	SEM: standard error of the mean
i.v.: intravenously	TNF: tumor necrosis factor
IC: interstitial cells	Trks: tyrosine kinase receptors
IC-IM: interstitial cells – intramuscular	TRP: transient receptor potential
IC-LP: interstitial cells – lamina propria	TTX: tetrodotoxin
IFO: ifosfamide	UDIF: urothelium derived inhibitory factor
IL: interleukin	UDP: uridine 5'-diphosphate
IP3: inositol trisphosphate	UTP: uridine 5'-triphosphate
IUS: internal urethral sphincter	

CHAPTER 1: Introduction

1.1 RATIONALE

Cyclophosphamide (CPO) and ifosfamide (IFO) are cytotoxic drugs used extensively in the treatment of cancer and autoimmune diseases (Furlanut and Franceschi, 2003, Brode and Cooke, 2008). A major limiting factor in the use of CPO and IFO is the resulting bladder toxicity which can manifest as urinary frequency, feelings of incomplete bladder emptying, dysuria, urgency, pain and in some cases life threatening hemorrhagic cystitis (Coggins et al., 1960, Bryant et al., 1980, Boddy and Yule, 2000, Furlanut and Franceschi, 2003, Klastersky, 2003, Korkmaz et al., 2007).

The urotoxicity of CPO and IFO is thought to be due to the formation of a metabolite called acrolein which is excreted in the urine and as a result comes into direct contact with the epithelial lining of the bladder (urothelium) (Dore et al., 1989, Boddy and Yule, 2000, Batista et al., 2006). The mechanisms of cytotoxic action in the urothelium are not fully understood although overproduction of reactive oxygen and nitrogen species (ROS/RNS), and depletion of antioxidant defences are believed to play a causal role (Korkmaz et al., 2007). Normal bladder function is dependent on the release of mediators from the urothelium induced by stretch during bladder filling and changes in the release of these factors are associated with bladder dysfunction (Kumar et al., 2010). Sensory nerves in the bladder are also imperative for normal bladder function. They sense chemical and mechanical changes and relay information about bladder fullness to the central nervous system, ultimately controlling the storage and micturition reflexes of the bladder (Kanai and Andersson, 2010). Disruption of afferent signalling can lead to storage and/or voiding dysfunction as well as pain.

The following review will cover the anatomy and physiology of the healthy bladder, detailed information about the drugs CPO and IFO and the known effects of these drugs on bladder function. This will highlight the gaps in the current literature and introduce the rationale and aims of this thesis.

1.2 SIGNIFICANCE

CPO and IFO are commonly used to treat a variety of malignant and non-malignant neoplastic diseases as well as being used as immunosuppressive agents in bone marrow transplantation and chronic autoimmune disorders (Lawson et al., 2008, de Jonge et al., 2005, Buda et al., 2003, Bergh, 2003, Nichols, 1995, Sladek, 1988). As these drugs are used in numerous therapeutic situations it is difficult to assess how many patients are treated with CPO or IFO each year. However, it is clear that the number of patients which may be affected by the toxicities of these drugs is substantial. CPO and IFO toxicity can adversely affect both short term and long term patient quality of life, can be life threatening, may limit the dose and duration of treatment possibly affecting treatment outcomes and may contribute to both the medical and nonmedical costs of treatment (Hensley et al., 1999).

It has been found that between 2-40% of CPO or IFO treated patients develop hemorrhagic cystitis (Hader et al., 1993, Takamoto et al., 2004). Incidence of micturition pain after treatment with CPO has been reported to be 19.6% and the incidence of the feeling of incomplete emptying of the bladder to be 15.2% (Fukuoka et al., 1991). Mortality rates of 2% to 4% have been reported for patients who develop severe bladder haemorrhage (Hensley et al., 1999). These adverse effects not only affect the tolerability of CPO and IFO treatment but also affect the ongoing quality of life of the patient. Furthermore, it has been estimated that the economic cost of managing CPO toxicities (not limited to urotoxicity) is between AU\$1,600 - \$6,500 per patient depending on country and chemotherapy regimen (Herold and Hieke, 2002).

During 2011 in Australia, a total of 45,168 out-patient scripts were filled for CPO and 2,025 for IFO at a total government cost of AU\$2.7million (Mabbott et al., 2013). A common uroprotectant used with CPO and IFO is mesna and a total of 2,230 out-patient scripts were filled in Australia in 2011 at a cost of AU\$344,778 (Mabbott et al., 2013). The low number of out-patient mesna prescriptions is thought to be due to the likelihood it is administered in hospitals, its cost, as well as the mixed reports on its efficacy. These costs are for pharmaceuticals prescribed to out-patients only while the number of prescriptions of CPO and IFO to patients in public hospitals is unknown. Another study places the in-hospital costs of mesna at US\$1500 per patient (Ballen et al., 1999). Furthermore, time spent in hospital and the cost of treatment would be increased in the event that treatment is stopped or delayed due to toxicities and to treat the toxicities themselves (Malliti et al., 2003). Accordingly, these figures are only suggestive of the significant costs associated with prevention and treatment of CPO and IFO urotoxicity. In addition, the cost of discomfort, pain and an overactive bladder on

quality of life after treatment cannot be represented in monetary values. A better understanding of what changes are occurring in the bladder as a result of CPO and IFO toxicity may lead to identification of possible targets for preventing or treating the adverse effects of these drugs as well as increased drug toleration and better treatment outcomes.

1.3 BLADDER

The urinary bladder is a hollow, muscular organ that temporarily stores urine which is formed by the kidneys and transported to the bladder via paired tubes called ureters. On emptying the bladder, urine passes through the urethra which conducts the urine to the exterior of the body in a process termed urination or micturition. Micturition involves contraction of the muscular bladder which forces urine through the urethra and out of the body (Martini and Nath, 2009).

Anatomy

The urinary bladder has several different regions, as shown in **Figure 1.1**, including an apex (dome), a base (trigone), a superior surface and two inferolateral surfaces (Drake et al., 2005). Each of the two ureters enters the bladder at one of the upper corners of the trigone while the urethral entrance lies at the lower corner of the trigone (Drake et al., 2005, Martini and Nath, 2009). The trigone acts as a funnel that channels urine into the urethra when the bladder contracts (Martini and Nath, 2009). The region surrounding the urethra entrance is known as the neck of the bladder. The control of continence at this section of the bladder is involuntary and the anatomy involved differs between males and females (Martini and Nath, 2009). Males have a well-defined ring of smooth muscle fibres between the base of the bladder and the upper border of the prostate (Jung et al., 2012). In females, there is not a well-defined ring of smooth muscle and it is the smooth muscle of the bladder neck and urethra that contribute to continence (Ashton-Miller and DeLancey, 2007). However, for the purpose of this thesis, both the male and female anatomy contributing to continence at the bladder neck will be referred to as the internal urethral sphincter (IUS). In both males and females, where the urethra passes through the urogenital diaphragm, there is a circular band of skeletal muscle which forms the external urethral sphincter (EUS). The EUS has resting tone and must be voluntarily relaxed to allow micturition (Martini and Nath, 2009).

Figure 1.1 has been removed due to Copyright restrictions.

Figure 1.1: The anatomy of the urinary bladder (adapted from (McKinley and O'Loughlin, 2008)).

The bladder wall consists of an outer serosal layer, a muscle layer (detrusor) and an inner urothelial/lamina propria layer (**Figure 1.1**). The inner lining of the bladder has been given several names including the mucosa, the urothelium/suburothelium or the urothelium/lamina propria and much international debate has surrounded which terminology accurately describes this layer. For clarity, throughout this thesis, the term urothelium/lamina propria will be used to collectively refer to the bladder layers of the urothelium, basement membrane and lamina propria (LP).

The urothelium is a layer of transitional epithelium and below it, separated by the basement membrane, is the LP which consists of blood vessels, nerves and interstitial cells (Guedes et al., 2008, Birder et al., 2010b). The urothelium/LP is loosely attached to the underlying smooth muscle and is usually thrown into folds, or rugae, that disappear as the bladder fills. However, the urothelium/LP of the trigone region is smooth, thick and firmly attached to the smooth muscle (Drake et al., 2005, Martini and Nath, 2009). The detrusor is formed by a circular layer of smooth muscle between an inner and outer layer of longitudinal smooth muscle. Contraction of the detrusor is responsible for compression of the bladder and expulsion of urine into the urethra (Martini and Nath, 2009).

As the urothelial layer is the outermost layer of the bladder it comes into contact with urine and any toxins present in the urine. Accordingly, it is this layer of cells that is of particular interest in the study of CPO and IFO induced urotoxicity and it will be discussed in more detail below.

Regulation of bladder function

Control of urine storage and micturition depends on coordination between the bladder muscle and the bladder outlet (the bladder neck, IUS and the EUS) (Sugaya et al., 2005). During micturition the bladder muscle contracts while the neck and the sphincters relax. Accordingly, during urine storage the neck and the sphincters contract while the bladder muscle remains inactive. The coordination of these opposing actions is regulated by a complex neural system in the brain, spinal cord and peripheral ganglia (Yoshimura et al., 2014). The peripheral nerves that control bladder function include the sympathetic, parasympathetic, and somatic nerves (Andersson, 1993, de Groat et al., 1993, Sugaya et al., 2005). The preganglionic sympathetic nerves emerge from the thoracolumbar spinal cord (T11-L2) and synapse with postganglionic nerves in the inferior mesenteric ganglion travelling mainly via the hypogastric nerve. The preganglionic parasympathetic nerves from the sacral spinal cord (S2-S4) join the pelvic nerves to synapse with postganglionic neurones in the terminal ganglia (pelvic ganglia and intramural ganglia within the bladder wall). While the somatic nerves also emerge from the sacral spinal cord (S2-S4) and join the pudendal nerve (Yoshimura et al., 2014). These nerves also have afferent fibres which carry sensory information from the bladder to the spinal cord.

Bladder voiding is primarily due to the parasympathetic efferent nerves which provide sustained excitatory cholinergic inputs to the bladder smooth muscle and nitric-oxide-mediated inhibitory inputs to the IUS (de Groat et al., 1993, Sugaya et al., 2005). Bladder filling is primarily controlled by the sympathetic nerves which provide noradrenergic inhibitory inputs to the bladder smooth muscle and excitatory inputs to the IUS (Andersson, 1993, de Groat et al., 1993, Sugaya et al., 2005). Control of the EUS is due to somatic cholinergic nerves which excite the muscle and must be voluntarily inhibited to allow bladder voiding (de Groat et al., 1993). The afferent nerves sense chemical and mechanical changes in the bladder and relay information about bladder volume during storage and the amplitude of contractions during voiding to the central nervous system (Kanai and Andersson, 2010). **Figure 1.2** illustrates the innervation of the bladder.

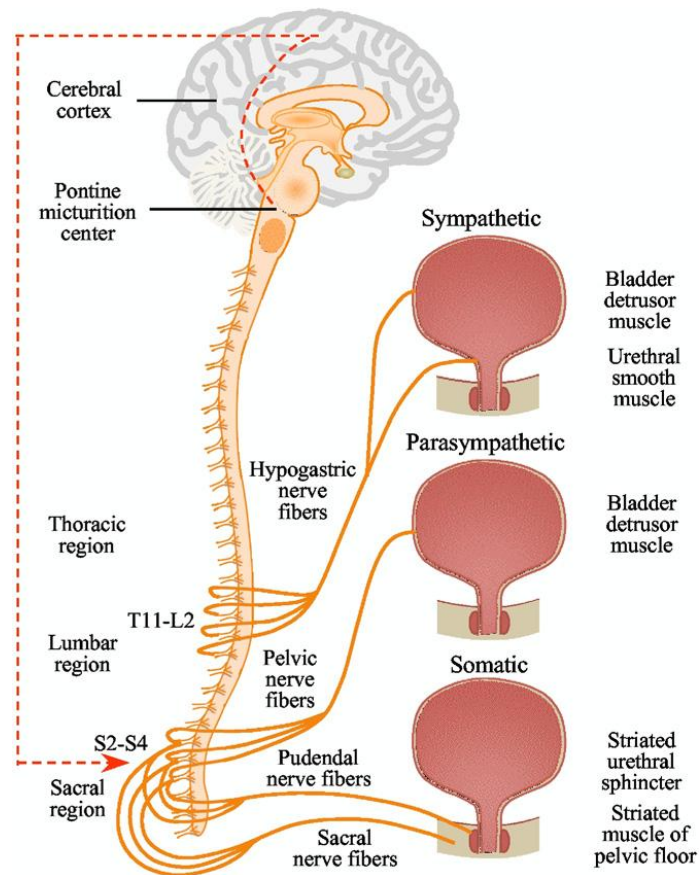


Figure 1.2: The innervation of the human lower urinary tract. Coordination between the detrusor and the bladder neck/urethra is mediated by sympathetic, parasympathetic and somatic nerves. Primary cell bodies of pelvic and pudendal afferents nerves are contained in lower lumbar and sacral dorsal root ganglia (DRG), and afferents in the hypogastric nerve are found in upper lumbar DRG (from (Kanai and Andersson, 2010) reproduced with permission from the publisher).

Efferent Nerves

Somatic Supply

Somatic supply to the bladder is via motor neurones in the ventral-ventromedial part of the anterior horn of S2 to S4 (nucleus of Onufrowicz). These neurones join the pudendal nerve and innervate the EUS. The somatic nerves provide cholinergic excitatory inputs to the striated muscle of the EUS which must be voluntarily inhibited to allow micturition (Sugaya et al., 2005).

Parasympathetic Supply

The majority of nerve fibres of the parasympathetic system release acetylcholine (ACh) which acts on nicotinic and muscarinic receptors. However, non-adrenergic, non-cholinergic (NANC) transmitters are often co-released along with ACh.

Early studies by Elliot, (1907) demonstrated that parasympathetic nerve stimulation in the cat resulted in relaxation of the IUS and contraction of the bladder muscle to cause micturition. It

has since been established that nitric oxide (NO) released from parasympathetic nerves is responsible for the relaxation of the IUS (Andersson et al., 1991) and ACh acting via muscarinic receptors is largely responsible for contraction of the detrusor (Clark and Ursillo, 1956). Most species studied also have an atropine resistant, NANC contribution to bladder contraction. Burnstock et al., (1972) were the first to provide evidence that adenosine 5'-triphosphate (ATP) was the transmitter responsible for the NANC contractions in rat and guinea pig bladder. Each of these transmitters is discussed further below.

ACh

Detrusor contractions of dog and rabbit bladder, elicited by electrical field stimulation, were largely abolished by the muscarinic receptor antagonist atropine (Clark and Ursillo, 1956). In the same study, it was found that direct stimulation of muscarinic receptors with ACh also resulted in contraction of the detrusor muscle. Subsequent studies in possum (Burnstock and Campbell, 1963) and rat (Vanov, 1965) also demonstrated that detrusor contractions were largely due to ACh release from pelvic parasympathetic nerves.

There are five subtypes of muscarinic receptor including M1-M5 (Caulfield and Birdsall, 1998). As shown in **Figure 1.3**, M1, M3 and M5 receptors couple to Gq/11 proteins that, once activated, stimulate phospholipase C (PLC) which leads to an up-regulation of the second messengers inositol trisphosphate (IP3) and diacylglycerol (DAG) (Caulfield and Birdsall, 1998). IP3 mobilises intracellular calcium (Ca^{2+}) stores and DAG activates the enzyme protein kinase C which phosphorylates cellular proteins and causes the influx of extracellular calcium to induce a response. These responses cause an excitatory effect in the cell and should manifest as a contraction. M2 and M4 receptors cause contraction indirectly by inhibiting the relaxatory effect of noradrenaline (NA). Specifically, M2 and M4 receptors are coupled to Gi/o proteins which inhibit adenylate cyclase (Peralta et al., 1988) resulting in a reduction of 3'-5'-cyclic adenosine monophosphate (cAMP) (Uchiyama and Chess-Williams, 2004) as shown in **Figure 1.4**.

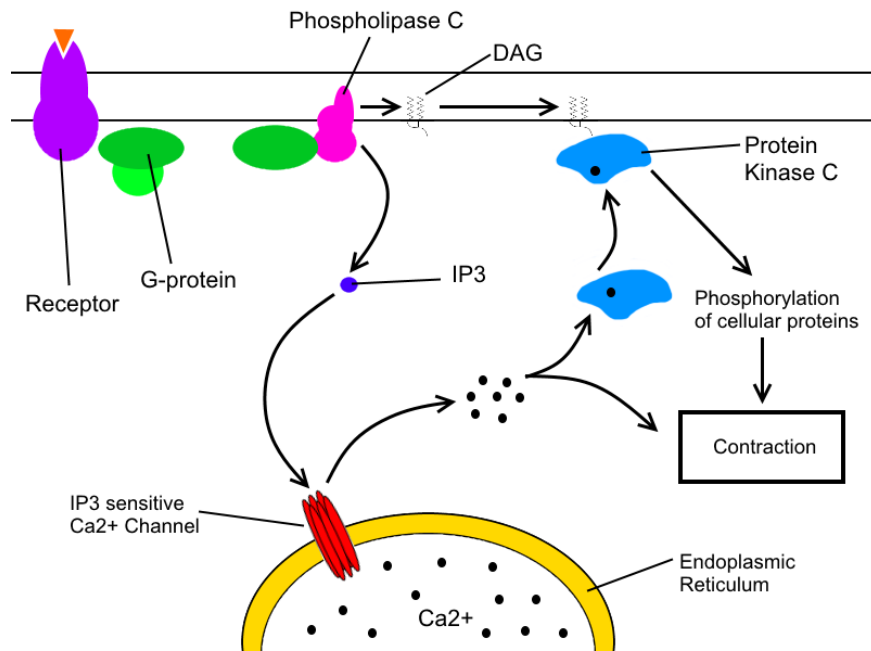


Figure 1.3 Cellular signalling in a smooth muscle cell. Receptors mediating activation of phospholipase C (PLC) include muscarinic receptors M1, M3 and M5 and α_1 -adrenoceptors. DAG = Diacylglycerol, IP₃ = inositol triphosphate.

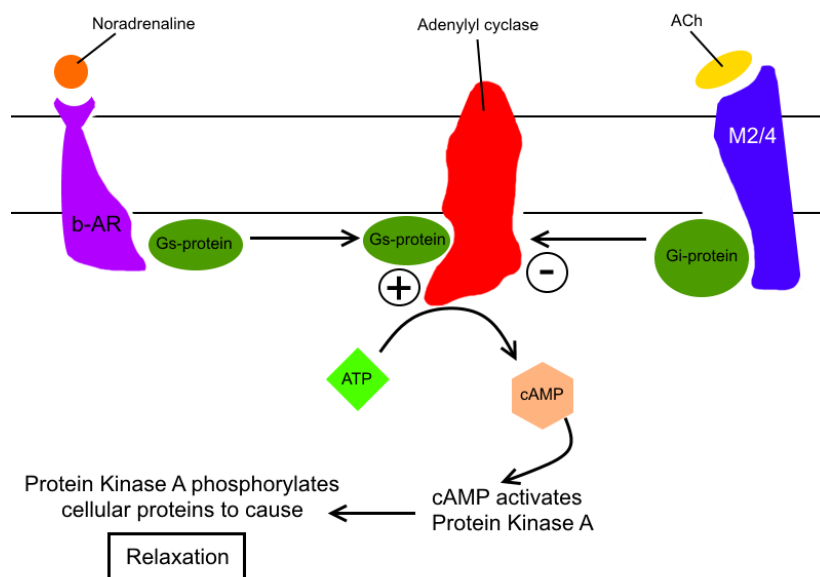


Figure 1.4 Cellular mechanism of muscarinic receptors M2 and M4 and β -adrenoceptor stimulation in a smooth muscle cell.

Northern blot and reverse transcription polymerase chain reaction (RT-PCR) studies have identified mRNA for only the M2 and M3 receptors in pig bladder (Maeda et al., 1988) and human bladder (Yamaguchi et al., 1996). Receptor immunoprecipitation studies have also identified the presence of only the M2 and M3 receptor protein in guinea-pig, rabbit and human bladder with the M2 receptor density being 3 times greater than that of the M3 receptor (Wang et al., 1995). However, direct contraction of the bladder detrusor has been shown to be

mediated via M3 receptors in all species so far examined (for review see (Chess-Williams, 2002)) including the human bladder (Fetscher et al., 2002).

The role of M2 receptors remains unclear and conflicting results have been reported. The M2 receptors have been shown to be functional at second messenger level causing a decrease in cAMP levels in human detrusor smooth muscle following stimulation with the muscarinic agonist carbachol (Harriss et al., 1995). Receptor manipulation studies have been used to investigate the role of M2 receptors in pig bladder whereby inactivation of the M3 receptors and elevation of cAMP levels with a β -adrenoceptor agonist resulted in muscarinic agonists eliciting a response at least partially mediated by the M2 receptors (Yamanishi et al., 2002a). As discussed further below, stimulation of sympathetic nerves results in relaxation of the bladder detrusor via β -adrenoceptor activation leading to an increase in cAMP. Therefore it has been proposed that stimulation of M2 receptors during parasympathetic activation may switch off the sympathetic inhibitory mechanism resulting in greater contraction and emptying of the bladder (Hegde et al., 1997, Yamanishi et al., 2002b).

Prejunctional muscarinic receptors have been identified in the bladder of several species which alter the release of neurotransmitters from the nerve endings (Somogyi and de Groat, 1999, de Groat and Yoshimura, 2001). Tobin & Sjogren (1998) showed that stimulation of prejunctional M1 receptors was responsible for increased release of ACh from parasympathetic nerves only at high frequency nerve stimulation of rabbit urinary bladder, while stimulation of prejunctional M2 receptors was responsible for decreased ACh release at both low and high frequency stimulation. Similar M1 facilitatory effects were also observed in rat bladders (Somogyi et al., 1994). Accordingly, it has been suggested that inhibitory M2 receptors are activated during short periods of low-frequency nerve activity and thereby suppress cholinergic transmission during urine storage (Somogyi and de Groat, 1999, de Groat and Yoshimura, 2001). On the other hand, M1 auto feedback receptors are activated during prolonged, high-frequency nerve firing that would occur during voiding, and therefore are thought to provide an amplification mechanism to promote complete bladder emptying (Somogyi and de Groat, 1999, de Groat and Yoshimura, 2001). In addition, M2 and M4 receptors are present on sympathetic nerves and their stimulation causes decreased release of noradrenaline (Mattiasson et al., 1984, Mattiasson et al., 1987, Trendelenburg et al., 2005).

Muscarinic receptor stimulation in the bladder has been shown to release a diffusible mediator capable of inhibiting smooth muscle contraction (Fovaeus et al., 1999). The urothelium has since been identified as the source of this unidentified factor which can inhibit contraction of the

underlying detrusor muscle in response to muscarinic receptor stimulation (Hawthorn et al., 2000). The urothelium will be discussed in more detail below.

Acetylcholine also acts at nicotinic receptors which are present throughout the body including at neuromuscular junctions between somatic motor neurones and skeletal muscle as well as in the ganglia of the autonomic nervous system. The nicotinic acetylcholine receptor family is currently known to consist of at least 17 different subunits (α 1–10, β 1–4, γ , δ and ϵ) which form pentameric channels that can be categorised into two different groups; neuronal nicotinic receptors (consisting of α 2–10 and β 2–4 subunits) and muscle nicotinic receptors (consisting of α 1-, β 1-, γ , δ and ϵ -subunits) (Lindstrom et al., 1996, Mamalaki and Tzartos, 1994). When nicotinic receptors are stimulated they undergo a conformational change and become cation-selective pores through the cell membrane. The pore allows movement of potassium (K^+), Ca^{2+} and sodium (Na^+) causing depolarisation of the cell and resulting in either muscle contraction or nerve depolarisation (**Figure 1.5**).

Nicotinic receptors are mediators of fast synaptic transmission in both the parasympathetic and sympathetic autonomic ganglia in the bladder (De Biasi, 2002). A study by Beckel et al., (2006) has also indicated a role for nicotinic receptors in bladder reflex function. Using RT-PCR and western blot analysis, the study detected mRNA in the urothelium for several nicotinic receptor subunits known to form functional receptors. The receptors were shown to be functional through calcium imaging studies while stimulation of these receptors in cultured urothelial cells with nicotine increased intracellular calcium.

However, a more recent study has demonstrated that the urothelium of the rat contains two subtypes of nicotinic receptor mediating distinct effects (Beckel and Birder, 2012). Specifically, these effects include inhibition of bladder reflexes via α 7 receptors and excitation of reflexes via α 3-containing receptors. These effects also appeared to be due to regulation of mediator release from the urothelium. Specifically, the excitatory response to α 3 receptor stimulation could be blocked by the purinergic antagonist PPADS suggesting nicotinic receptor regulation of ATP release which then enhances afferent activity (Beckel and Birder, 2012).

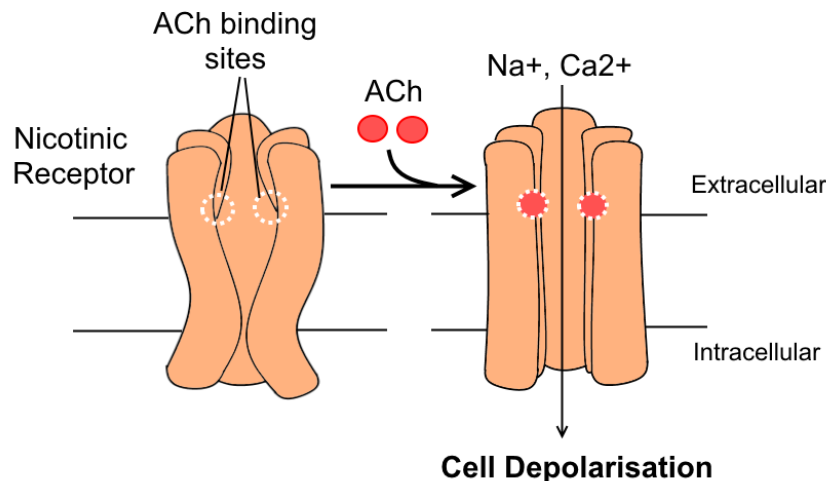


Figure 1.5: Nicotinic receptor stimulation by acetylcholine causes an influx of sodium and calcium and cellular depolarisation.

ATP

Studies have demonstrated that exogenous ATP causes contractions of the bladder which mimic the NANC contractions in response to electrical field stimulation (EFS). Desensitisation of receptors with purinergic agonists suppressed ATP-induced and NANC contractions and both were enhanced by ATPase inhibitors which reduce the breakdown of ATP (Burnstock et al., 1972, Ralevic and Burnstock, 1998).

ATP is a purine and acts on purine receptors which can be divided into two main families, adenosine receptors called P1 receptors, and P2 receptors recognising primarily ATP, adenosine 5'-diphosphate (ADP), uridine 5'-triphosphate (UTP), and uridine 5'-diphosphate (UDP) (Ralevic and Burnstock, 1998). The P2 receptors divide into two families of ligand-gated ion channels and G protein-coupled receptors termed P2X and P2Y receptors respectively. P2X binds primarily ATP while P2Y binds ADP, UTP and UDP (Ralevic and Burnstock, 1998). There are seven ionotropic P2X receptors, P2X1-7. Upon activation, P2X receptors become permeable to monovalent cations as well as a relatively high permeability to calcium. P2X1 and P2X3 show fast desensitisation to agonists (within 1-2 seconds), P2X2, 4 and 5 show slow desensitisation to agonist application (minutes) and P2X7 does not desensitise (North, 2002).

Various species are known to express purinergic receptors in the bladder including human, mouse, rat, cat and guinea pig (Burnstock and Kennedy, 1985, Lee et al., 2000, Vial and Evans, 2000, O'Reilly et al., 2001, Birder et al., 2004). Specifically, it has been demonstrated that the P2X1 subtype is primarily responsible for mediating the NANC contraction of the mouse detrusor (Vial and Evans, 2000). However, excitatory transmission in the normal human bladder appears to be entirely cholinergic (Sjogren et al., 1982, Sibley, 1984, Kinder and Mundy,

1985, Chen et al., 1994), with an atropine-resistant component appearing only in bladders from patients with bladder pathologies such as interstitial cystitis and is thought to be due to ATP (Sjogren et al., 1982, Palea et al., 1993). The P2X1 is also the most abundant P2X receptor subtype in the adult human bladder which may be responsible for the ATP response in disease (O'Reilly et al., 2001).

It has also been demonstrated that the P2X3 subtype is present on suburothelial nerve plexi and urothelium (Birder et al., 2004, Cockayne et al., 2000, Lee et al., 2000). In addition, P2Y receptors have been identified on cat, rat and guinea pig urothelial cells (Sui et al., 2006, Birder et al., 2004) and specifically the P2Y2 and P2Y4 subtypes (Chopra et al., 2008). The roles of purinergic receptors on the urothelium and suburothelial plexus are discussed below.

Nitric Oxide

Early studies in rabbit identified that nitric oxide (NO) was the neuronal messenger responsible for the relaxation of the urethra (Andersson et al., 1991). NO has a short half life and is very reactive so it cannot be stored or packaged in vesicles in nerves. Rather, NO is formed on demand by the hydroxylation of L-arginine to citrulline (Palmer and Moncada, 1989) which is catalysed by one of three isoforms of nitric oxide synthase (NOS) (Forstermann et al., 1994). Three types of NOS have been identified in the lower urinary track and the neural pathways controlling the bladder including endothelial NOS (eNOS), neuronal NOS (nNOS) and inducible NOS (iNOS) (Dokita et al., 1991, Andersson and Persson, 1994, Ehren et al., 1994a, Ehren et al., 1994b, Burnett et al., 1997, Lemack et al., 1999, Johansson et al., 2002, Masuda et al., 2002, Felsen et al., 2003). The three isoforms were named after the cells in which they were first isolated, purified and cloned (Forstermann et al., 1994). Despite their names, eNOS and nNOS are normal constituents of cells while the expression of iNOS is induced by certain cytokines or bacterial endotoxins (Moncada et al., 1991).

The pelvic ganglia of the parasympathetic system show NOS immunoreactivity (Vizzard et al., 1994). Studies in rat have shown that nNOS is most prominent in the parasympathetic postganglionic innervation of the urethra and non-existent in the innervations of the detrusor (Vizzard et al., 1994). Studies in rabbit, pig and rat have demonstrated that administration of L-arginine decreases the pressure of the IUS while NOS inhibitors, inhibit the relaxation of the IUS in response to neuronal stimulation (Andersson et al., 1992, Persson and Andersson, 1992, Persson et al., 1992, Kawahara, 1994, Bennett et al., 1995). Furthermore, both endogenous NO and stimulation of the parasympathetic nerves supplying the urethra elicits NO-dependent

urethral smooth muscle relaxation but does not relax the detrusor (Bennett et al., 1995, de Groat and Yoshimura, 2001, Persson et al., 1992).

In summary, the parasympathetic nerves provide excitatory cholinergic inputs (a role for purinergic transmitters in disease) to the bladder and have an inhibitory nitrenergic effect on the IUS (de Groat et al., 1993, Ralevic and Burnstock, 1998, Sugaya et al., 2005).

Sympathetic Supply

Sympathetic nerves release noradrenaline, which acts on adrenoceptors, and provides excitatory inputs to the bladder neck and the urethra, inhibitory inputs to the detrusor as well as both facilitatory and inhibitory inputs to parasympathetic ganglia (Andersson, 1993, de Groat et al., 1993, Sugaya et al., 2005).

Early studies by Burnstock and Campbell, (1963) demonstrated that the possum detrusor relaxed in response to both adrenaline and noradrenaline. Subsequent work in cats by Gjone (1965) and Edvardsen (1968) demonstrated that transection of the vesical sympathetic nerves resulted in increased spontaneous and reflex evoked contractions of the bladder. As a result it was proposed that the sympathetic fibres exert tonic inhibitory influences on the bladder (De Groat and Saum, 1972). Hamberger & Norberg (1965) found that adrenergic terminals in the bladder were located primarily in the intramural plexus, often in synaptic contact with parasympathetic ganglion cells suggesting that that sympathetic inhibition of bladder contraction may also result from inhibition of transmission of the parasympathetic excitatory pathway to the bladder. Furthermore, nerves immunoreactive for tyrosine hydroxylase, required in the production of noradrenaline and neuropeptide Y, a co-transmitter of noradrenaline, are rare in the detrusor however they are moderately frequent in the suburothelium (Birder et al., 2010a). Nerves immunoreactive for tyrosine hydroxylase and neuropeptide Y have also been found to synapse with presynaptic parasympathetic nerve terminals in the bladder where they affect ACh release (Birder et al., 2010a).

Noradrenaline

De Groat & Saum, (1972) demonstrated that stimulation of sympathetic nerves elicited an initial contraction of the bladder of short duration followed by a prolonged relaxation below control levels. In addition, administration of catecholamines to the bladder depressed spontaneous and nerve-evoked contractions of the bladder, decreased the resting bladder tone and these effects were reduced or completely blocked by β -adrenergic blocking agents.

There are two types of adrenoceptor (AR) being the α - and β -ARs. Both α - and β -ARs are expressed in the bladder of various species including humans and rats (Walden et al., 1997, Hampel et al., 2002). There are two subtypes of α -adrenoceptor, α_1 and α_2 -ARs. Both subtypes are linked to G-proteins; α_1 -ARs are linked to a G_q -protein and α_2 -ARs are linked to a G_i -protein. When stimulated, the α_1 -ARs activate the G_q -protein which activates the enzyme PLC and causes the production of the second messengers IP3 and DAG (See **Figure 1.3**). The overall effect of these second messengers is generally an excitatory effect. When an α_2 -AR is stimulated it activates a G_i -protein which reduces cAMP production by inhibiting the enzyme adenylate cyclase. The G_i -protein also activates K^+ channels causing cell hyperpolarisation having an overall inhibitory effect on the cell. These receptors tend to be found on nerve terminals and their activation leads to inhibition of neurotransmitter release.

Walden et al., (1997) demonstrated that overall expression of α -ARs in the human bladder detrusor is low however it is the α_1 -AR subtype which is predominant. In most species, stimulation of the α_1 -ARs with high concentrations of agonist results in detrusor contraction (Andersson, 1993). Similarly, in normal human detrusor, selective stimulation of the α -ARs produces small and variable contractile responses (Andersson, 1993).

Three β -AR subtypes exist including β_1 , β_2 , β_3 . Similar to α -ARs the β -ARs are also linked to a G-protein (see **Figure 1.4**). Li et al. (2004) observed that β_1 and β_3 -ARs activated a $G_{i/o}$ -protein subunit stimulating the enzyme guanylate cyclase and increasing 3',5'-cyclic guanosine monophosphate (cGMP) production; whereas the β_2 -ARs activate both the G_s and the $G_{i/o}$ -protein subunits and stimulate adenylate cyclase and guanylate cyclase producing cAMP and cGMP respectively. These second messengers have various effects within the cell but generally result in relaxation of smooth muscle.

Early studies investigating the β -ARs in bladder detrusor showed that the β_2 -ARs predominate (Andersson, 1993). However, in some species, such as guinea pig, it was shown that the β_1 -ARs mediate relaxation despite the β_2 -AR subtype predominating (Andersson, 1993). Human detrusor expresses β -ARs which have functional characteristics of a subtype other than β_1 - or β_2 -ARs. These receptors mediate a relaxation that can be blocked by a β -AR antagonist (propranolol) but not a β_1 -AR selective antagonist (practolol) or a β_2 -AR selective antagonist (butoxamine) suggesting the β_3 -AR is responsible for the relaxation (Nergardh et al., 1977, Larsen, 1979). Since then various methods such as RT-PCR, *in situ* hybridization and *in vitro* contractile experiments have been used to demonstrate that human detrusor can express all three β -AR subtypes (Andersson and Arner, 2004). However, β_3 -AR agonists are the most

effective at relaxing the human detrusor (Badawi et al., 2007, Yamaguchi, 2002, Igawa et al., 2001, Takeda et al., 1999, Igawa et al., 1999) suggesting that the most important β -AR in bladder relaxation is the β_3 -AR, at least in humans (Andersson and Arner, 2004). β_3 -ARs have also been demonstrated in the detrusor of the pig (Yamanishi et al., 2002c, Yamanishi et al., 2002d) as well as the rat (Clouse et al., 2007).

Adrenoceptors have also been found on parasympathetic nerves where they affect release of ACh. Specifically, release of ACh from parasympathetic nerves can be increased by stimulation of facilitatory α_1 -ARs and decreased by stimulation of inhibitory α_2 -ARs (Keast et al., 1990, Tobin and Sjogren, 1998). In addition, α_2 -AR's have also been found prejunctionally on sympathetic nerves and their activation also results in decreased noradrenaline release thereby acting as a negative feedback loop (Mattiasson et al., 1984, Mattiasson et al., 1987).

Other Neurotransmitters

A wide range of other transmitters have been proposed in human bladder including vasoactive intestinal peptide, calcitonin gene-related peptide (CGRP), tachykinins, substance P, bradykinin, pituitary adenylate cyclase activating peptide (PACAP), adenosine, prostaglandins, serotonin, cannabinoids and neurotrophic factors (reviewed by de Groat & Yoshimura (2001). However, these will not be covered as the purpose of this review is to provide an overview of the function of the bladder and the main neurotransmitters have been discussed above.

Table 1.1: Summary of receptor expression in the detrusor

Receptor	Subtype present	Species	Reference
Muscarinic	M2 & M3	Pig	(Maeda et al., 1988, Sellers et al., 2000)
		Human, guinea pig, rat & rabbit	(Wang et al., 1995)
		Human	(Yamaguchi et al., 1996, Harriss et al., 1995)
Purinergetic	P2X1	Mouse	(Vial and Evans, 2000)
	P2X1 & P2X2	Cat	(Birder et al., 2004)
	P2X1, (P2X2, P2X5, P2X6)	Rat	(Lee et al., 2000)
Adrenergic	α_1 - AR	Rat, monkey and human	(Walden et al., 1997)
	β_3 -AR	Human	(Larsen, 1979, Nergardh et al., 1977)
	β_3 -AR	Pig	(Yamanishi et al., 2002c, Yamanishi et al., 2002d)
	β_3 -AR	Rat	(Clouse et al., 2007)
	β_3 -AR	Mouse	(Deba et al., 2009)

Afferent Nerves

The bladder contains a complex network of afferent axons projecting mostly via the pelvic nerve but also via the hypogastric and pudendal nerves to the dorsal root ganglia of the upper sacral and lumbar levels of the spinal cord (Gabella and Davis, 1998). Pelvic and pudendal nerves predominantly have their cell bodies in the sacral spinal cord (S1-S4) whilst hypogastric nerves project to the thoracolumbar segments (T10-L2) of the spinal cord.

Location of Afferent Nerves

In both humans and animals afferent nerves have been found both suburothelially and in the detrusor muscle (Gosling and Dixon, 1974, Dixon et al., 1983, Gabella and Davis, 1998, Wakabayashi et al., 1993, Gabella, 1999). Specifically, afferent axons have been found in distinct regions including (1) at the base of the urothelium, where they form a suburothelial plexus, (2) inside the urothelium, (3) on blood vessels, and (4) along muscle bundles (Gabella and Davis, 1998, Wakabayashi et al., 1993, Morrison, 1999). The suburothelial plexus was

found to be thickest in the neck of the bladder and initial portion of the urethra and progressively less dense in the adjacent regions. Whereas the afferent innervation of the smooth muscle was diffuse and appeared uniform throughout the bladder.

Pelvic sensory nerve terminals are uniformly distributed to all areas of the detrusor and urethra with receptive fields in the mucosa and serosa of the body, base, and at the ureterovesical junction. The lumbar afferent nerve terminals are most frequent in the trigone and sparse in the bladder body with receptive fields in the mucosa and serosa and associated with blood vessels (Andersson, 2002, Jung et al., 2012, de Groat and Yoshimura, 2009). Pelvic afferent nerves exhibit dynamic responses to stimulation and adapt to maintained stimulus, whereas lumbar nerves do not give dynamic responses or adapt to maintained forces (de Groat and Yoshimura, 2009). The pelvic nerves are the primary pathway for sensations related to filling, voiding, discomfort and pain (de Groat, 1997) and is the primary pathway considered in this thesis.

Classification of Afferent Nerves

There are two main types of afferent fibres in the bladder, myelinated A- δ fibres and unmyelinated C-fibres (Kanai and Andersson, 2010, Birder et al., 2010a, Gabella and Davis, 1998). Unmyelinated C-fibres are more common than myelinated A- δ fibres comprising approximately 60-70% of total nerve fibres (Shea et al., 2000, Vera and Nadelhaft, 1990). The afferent nerves can be classified based on their size, function, content or sensitivity to various substances. Functional classification can be based on conduction velocity and their response to mechanical or chemical stimuli.

The A- δ fibres are larger in diameter and conduct action potentials more rapidly (~11.0m/s) than the C-fibres (~1.7 m/s) (Sengupta and Gebhart, 1994, Shea et al., 2000). Bladder afferents can also be characterised as stretch-sensitive mechanoreceptors, stretch-insensitive chemoreceptors and silent afferents that do not respond to distension or chemical stimuli but may become mechanosensitive during inflammation (Shea et al., 2000, Zagorodnyuk et al., 2006, Habler et al., 1990). However, Shea et al., (2000) also reported a population of fibres that were both chemosensitive and mechanosensitive responding to both stretch and an inflammatory mixture. In the mouse pelvic nerve, four classes of mechanosensitive bladder afferents (muscular, urothelial, muscular/urothelial, and serosal) have been identified based on responses to receptive field stimulation by probing, stretch or urothelial stroking (Jung et al., 2012). Similar classes of nerves have also been reported in the guinea pig bladder including muscle and mucosal mechanoreceptors (Zagorodnyuk et al., 2006). These classes are discussed further in Chapter 5.

The mechanical threshold at which the afferent fibres become active can also be used to classify fibres as low threshold or high threshold. Low threshold fibres become active at intravesical pressures below 15mmHg and high threshold fibres become active at pressures above 15mmHg (Daly et al., 2007, Rong et al., 2002, Shea et al., 2000). Low threshold fibres are mainly involved in normal bladder distensions and are more common than high threshold fibres which are involved in painful sensations (de Groat, 1997, de Groat and Yoshimura, 2009). Both A- δ fibres and C-fibres can have low or high thresholds (Sengupta and Gebhart, 1994, Shea et al., 2000).

Neurofilament is a cytoskeletal protein synthesised in A- δ fibres only and can be visualised by immunohistochemical labelling. In rats, approximately one third of bladder afferent neurones were immunoreactive to neurofilament (A- δ fibres) with the remaining two thirds being neurofilament poor (C-fibres) (Yoshimura et al., 1998). Other immunocytochemical studies have revealed that numerous neuropeptides are localised alone or together in most bladder afferent neurones including substance P, CGRP, vasoactive intestinal polypeptide, neurokinin A, PACAP, enkephalins, and cholecystokinin (de Groat and Yoshimura, 2001, Keast and De Groat, 1992). Axons containing these peptides are found throughout the bladder but are particularly dense in the suburothelial plexus (de Groat and Yoshimura, 2009). These peptides may function as transmitters at afferent terminals in the spinal cord or may be released in the bladder by noxious stimulation and cause plasma extravasation, vasodilation and alterations in smooth muscle activity (Morrison, 1999).

The majority of C-fibre afferents are sensitive to capsaicin and can be termed capsaicin sensitive primary afferent nerves whereas, very few A- δ fibres are sensitive to capsaicin (Szallasi and Blumberg, 1999, de Groat and Yoshimura, 2009). In response to treatment with capsaicin, C-fibres release CGRP, substance P, and PACAP in the bladder wall and can trigger inflammation (Maggi, 1995).

Receptor Expression

Afferent neurones also express various receptors which may be activated in an autocrine fashion or by other transmitters released from the urothelium or interstitial cells. These receptors include transient receptor potential (TRP) channels, purinoceptors, nicotinic, tachykinin, and prostanoid receptors (Andersson, 2002).

TRP channels are a group of cation channels that can be found on the surface of various human and animal cells. The TRP superfamily can be divided into seven sub families including the TRPC (canonical) and TRPM (melastatin), TRPV (vanilloid), TRPA (ankyrin), TRPP

(polycystin) and TRPML (mucolipin) families (Nilius et al., 2007). The TRPV subfamily is the most investigated of the subfamilies in the lower urinary tract. TRPV1 channels are widely distributed in bladder afferent nerve terminals and urothelium and are thought to play a role in normal and painful sensations in the bladder (Birder et al., 2002a). TRPV1, previously known as the vanilloid receptor type 1 or the capsaicin receptor, is a nonselective cation channel with a preference for calcium and can be activated by noxious stimuli, heat, protons, pH <5.9, capsaicin, and endogenous agonists, such as anandamide (Tominaga et al., 1998, Tominaga and Tominaga, 2005, Everaerts et al., 2008). The activation of these receptors in nerves initiates a complex cascade of intracellular events which results in excitation, desensitisation or neurotoxicity (Szallasi and Blumberg, 1999). In the rat bladder, most TRPV1 immunoreactive fibres co-expressed the neuropeptides substance P and CGRP (Ost et al., 2002). Another subtype, TRPA1, has been found to be expressed in C-fibre afferents as well as the urothelium and stimulation of these channels results in bladder hyperreflexia (Du et al., 2007b, Streng et al., 2008). The gaseous molecule hydrogen sulphide, which may be formed during infection or inflammation, is an activator of TRPA1 suggesting a possible role for this channel in inflammatory conditions (Streng et al., 2008).

It has been shown that of the seven P2X purinoceptors receptor subtypes, the P2X3 receptors are expressed on afferent neurones in the bladder and predominantly on small-diameter nociceptive neurones (Chen et al., 1995, Dunn et al., 2001). ATP, released by urothelial cells when stretched, is believed to act on these receptors to increase micturition reflexes and convey information of bladder fullness (Cockayne et al., 2000, Vlaskovska et al., 2001, Burnstock, 2001). The role of the urothelium is discussed in more detail below.

Nicotinic receptors are present in sensory neurones and nicotine can induce inwards currents in rat dorsal root ganglia (DRG) neurones (Haberberger et al., 2004). Furthermore, mRNA expression of various nicotinic receptor subtypes has been recently shown in bladder sensory nerves in mice (Nandigama et al., 2013). In particular, the $\alpha 3$ -containing subtype was expressed in 69% of sensory nerves.

Prostanoids, such as prostaglandins, are synthesised in bladder smooth muscle and urothelium by cyclooxygenase (COX) enzymes (Maggi, 1992, Andersson, 1993). Two types of COX enzymes have been identified being COX-1, which is normally expressed and responsible for the production of prostanoids involved in physiological processes, and COX-2, which is induced by inflammatory stimuli. Synthesis can be induced by various stimuli including stretch of the detrusor muscle, injury, nerve stimulation, chemical mediators such as ATP and inflammatory

mediators (Andersson, 2002). Prostanoids, especially prostaglandin E₂ (PGE₂), have been implicated as endogenous mediators of bladder function and may act directly on bladder smooth muscle and/or indirectly as neuromodulators of efferent and afferent neurotransmission (Andersson, 2002). PGE₂ acts on the family of G-protein coupled EP receptors which includes 4 subtypes EP1-4. EP1 is coupled to Ca²⁺ mobilisation, EP3 inhibits adenylate cyclase and EP2 and 4 both stimulate adenylate cyclase (Beppu et al., 2011). PGE₂ has been shown to facilitate micturition and increase basal intravesical pressure in conscious rats (Ishizuka et al., 1995b, Ishizuka et al., 1995a). The EP1 and EP3 receptor subtypes appear to have a role in overactivity of the detrusor muscle, while EP4 receptors have been found in dorsal root ganglion sensory neurons and induce an increase in cAMP production and peptide release from nerves leading to nerve sensitisation (Kopp et al., 2004, Southall and Vasko, 2001, Beppu et al., 2011). It was suggested that this effect may be due to PGE₂ stimulated release of tachykinins from nerves in and/or immediately below the urothelium which initiate a micturition reflex by stimulating NK-1 and NK-2 receptors on the urothelium or possibly on the nerves themselves (Ishizuka et al., 1995b, Ishizuka et al., 1995a).

Mechanosensitivity

Mechanosensitivity refers to the ability of an afferent nerve to sense a mechanical force. In the bladder, mechanical information from afferent nerves is responsible for the micturition reflexes, the sensation of bladder fullness and the desire to void and controls activity in the parasympathetic and sympathetic efferent nerves to the lower urinary tract (Andersson, 2002). Exactly how the bladder afferents sense these mechanical forces is unknown. However, there are both direct and indirect ways in which this can occur.

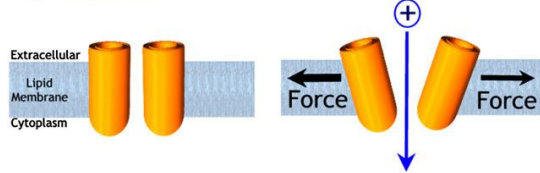
Direct

The purpose of mechanosensitivity is to rapidly transduce sensory information to the central nervous system. Accordingly, it has been thought that afferent nerves may have ion channels that can respond directly to mechanical stimuli to elicit an immediate response, without the need for second messengers or gene transcription (Araki et al., 2008). In the bladder this means a transduction channel must be sensitive to incremental increases in bladder pressure/muscle stretch producing a graded increase in frequency of the action potentials. There are two main theories about how mechanosensitive channels are activated (**Figure 1.6**). The first is via direct deformation of the channel due to membrane compression, expansion, thickness, or local curvature. The second hypothesis involves tugging on the channel protein by cytoskeletal or extracellular tethers (Gillespie and Walker, 2001, Hamill and Martinac, 2001). In either process,

mechanical stimuli changes the open probability of the channel and thereby alters the mechanosensory signal (Gillespie and Walker, 2001).

A) Direct channel gating

1) Bilayer tension



2) Tethered Channel

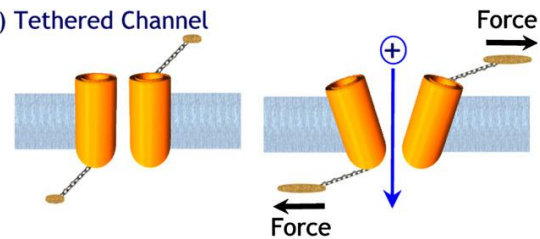


Figure 1.6: Proposed models by which a mechanotransduction channel is gated by mechanical stimuli (Brierley, 2010). Reproduced with permission of the publisher.

The exact identity of such a channel in bladder afferents is unknown, however some of the channels that have been identified in other mechanosensitive systems include the epithelial sodium channel (ENaC), acid sensing ion channel (ASIC), degenerin Na⁺ channels (Deg) and TRP channels (Gillespie and Walker, 2001). Recently, ENaC, ASIC 1 & 2 and TRPV1, TRPM8 and TRPA1 channels have been found in various parts of the urinary tract (Araki et al., 2008, Birder et al., 2001, Du et al., 2007a, Mukerji et al., 2006a, Nagata et al., 2005, Stein et al., 2004). However, given the diverse locations of these channels it is not clear whether they contribute to direct or indirect mechanisms of mechanosensitivity or both.

Indirect

Mechanotransduction may also occur indirectly through the interaction of other structures with the afferent nerves. Such interactions may include the release of mediators from the urothelium in response to stretch activating or sensitising afferent nerves or the ability of the interstitial cells to communicate electrically with the afferent nerves. As discussed above, the urothelium releases various mediators in response to stretch, with ATP thought to be a crucial mediator of mechanotransduction. The P2X3 receptors on sub-urothelial afferents appear to be particularly important as their absence leads to bladder hyporeflexia (Cockayne et al., 2000). Furthermore, in a study by Vlaskovska et al., (2001) the stretch induced activity in pelvic sensory nerves could be mimicked by ATP and $\alpha\beta$ mATP and attenuated by P2X3 antagonists as well as in P2X3 knockout mice. See **Figure 1.7** for an overview of this mechanism.

Interstitial cells (ICs) in the lamina propria may also play a role in the indirect mechanotransduction. ICs respond to ATP by firing Ca²⁺ transients (Fry et al., 2007, McCloskey and Gurney, 2002, Sui et al., 2006, Wu et al., 2004) potentially linking the urothelial ATP to afferent nerve excitation. The interaction between urothelium and ICs is discussed further below.

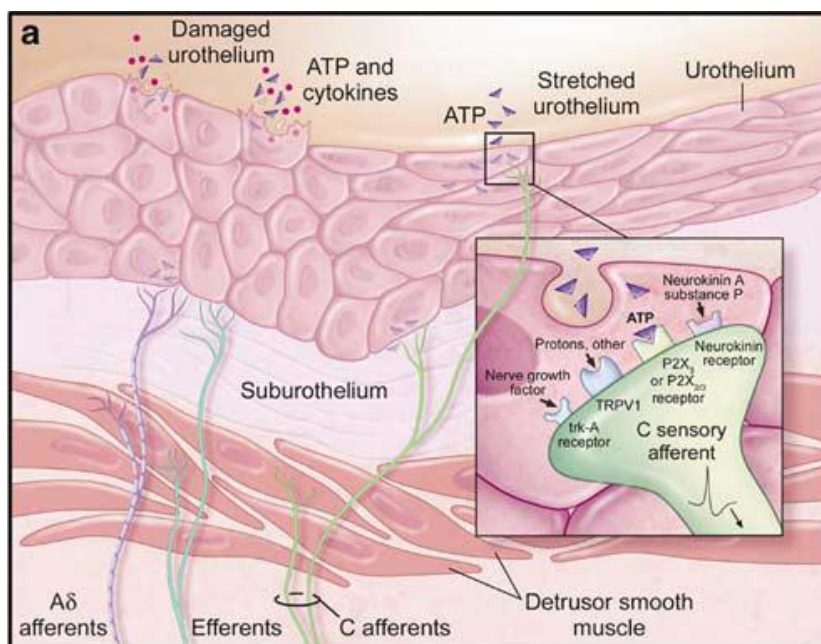


Figure 1.7: Schematic diagram showing the indirect role of ATP from the urothelium on P2X receptors on the peripheral terminals of A δ - and C-fibre bladder afferents, where it may convey mechanosensory and nociceptive information to the spinal cord (Ford et al., 2006). Reproduced with permission of the publisher.

Micturition Reflexes

Coordination of the storage of urine and micturition is mediated by multiple reflex pathways organised in the brain and spinal cord and are initiated by afferent input (de Groat and Yoshimura, 2001). Storage reflexes in the spinal cord involve the activation of mechanoreceptors on afferent nerves during bladder filling which result in firing in the cholinergic somatic efferent nerves to the EUS and in sympathetic adrenergic nerves to the IUS (de Groat and Yoshimura, 2001). Afferent projections to the brain terminate in various nuclei including the periaqueductal gray (PAG) which conveys information to a micturition centre in the dorsolateral pons, called the pontine micturition centre (PMC). When the bladder volume reaches the micturition threshold, the afferent activity activates the PAG which then conveys this information to the PMC to initiate the micturition reflex. The micturition reflex involves activation of the parasympathetic pathway to the bladder, inhibition of the spinal storage reflexes and results in bladder contraction, sphincter relaxation and bladder emptying (Yoshimura et al., 2008).

The PMC, is thought to serve as a relay centre or “on-off” switch, receiving information from the PAG as well as inhibitory and excitatory influences from areas of the brain rostral to the pons (de Groat and Yoshimura, 2001, Sugaya et al., 2005). Central nervous system regulation of voiding is further complicated by the fact that these pathways are also under voluntary control.

Higher brain centres can suppress excitatory signals from the PAG to the PMC during bladder filling to suppress the voiding reflex (Drake et al., 2010).

The PAG also transmits efferent information back to the bladder from the PMC to initiate voiding. The efferent signals inhibit the thoracolumbar sympathetic nucleus and the pudendal nerve nucleus in the sacral cord, resulting in relaxation of the IUS and EUS respectively as well as exciting the parasympathetic nucleus in the sacral cord inducing detrusor contraction and further IUS relaxation (Sugaya et al., 2005).

Myogenic & Spontaneous Tone

Myogenic activity is the ability of a smooth muscle cell to contract independent of external stimuli. Normally during the filling or storage stage there is no parasympathetic input to the bladder (de Groat, 1997) however, the bladder exhibits spontaneous phasic contractions or myogenic activity (Andersson and Arner, 2004). The spontaneous contractions demonstrated *in vitro* are resistant to tetrodotoxin (TTX) and cannot be blocked by hexamethonium, atropine, α - and β -ARs blockers or suramin, supporting a myogenic origin (Andersson and Arner, 2004). Some evidence suggests that the smooth muscle cells of the detrusor are electrically coupled by gap junctions and that these connections may be important in the initiation and maintenance of detrusor tone (John et al., 2003, Neuhaus et al., 2002, Wang et al., 2001). The myogenic contractile activity of the detrusor cells may be reinforced by mediators released from neurones and other sources such as the urothelium (Andersson and Arner, 2004). Spontaneous contractile activity is increased in conditions with detrusor overactivity (Andersson and Arner, 2004) and therefore its regulation is of interest in the bladder dysfunction seen after CPO or IFO treatment.

The urothelium/lamina propria also develops spontaneous, phasic contractions (Moro et al., 2011). Contraction of the lamina propria region was first demonstrated in rabbit urethra in response to various agonists and electrical field stimulation (Mattiasson et al., 1985, Zygmunt et al., 1993). The urothelial/lamina propria layer of the pig bladder has also been shown to exhibit contractile properties in response neurokinin A and muscarinic receptor stimulation despite the absence of smooth muscle (Sadananda et al., 2008). Furthermore, the spontaneous phasic contractions of the urothelial/lamina propria layer of the pig bladder become more frequent in response to stretch and muscarinic receptor stimulation (Moro et al., 2011). The increased contraction rate seen after stretch and muscarinic receptor stimulation was attributed to M3 receptor activation by endogenous ACh release. It has been suggested that the contractile

property of the urothelial/lamina propria region is due to the network of interstitial cells in the lamina propria (Sadananda et al., 2008).

Urothelium

Structure

As briefly mentioned above, the urothelium is the stratified squamous epithelial lining of the bladder and is composed of at least a basal cell layer attached to the basement membrane, an intermediate layer and a superficial layer (or apical layer) composed of large hexagonal cells known as umbrella cells (Birder et al., 2010c). These umbrella cells are interconnected by tight junctions and are covered nearly completely (70-80%) by crystalline hexagonal arrays of 16 nm proteins called uroplakins (**Figure 1.8**) (Liang et al., 2001, Sun, 2006, Birder et al., 2010c). Four types of uroplakins exist on the mammalian urothelium including UPIa, UPIb, UPII, and UPIII (Lee, 2011).

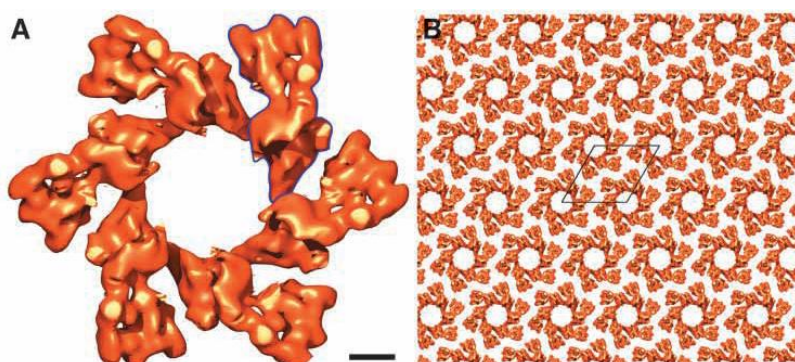


Figure 1.8 The 3D structure of the 16nm mouse uroplakin particle at 10 Å resolution. (A) A top view of the uroplakin particle. (B) A hexagonal crystalline array with one unit cell illustrated (from (Min et al., 2003) reproduced with permission of the publisher).

The outer surface of the urothelial apical cell membrane also includes a layer of glycosaminoglycans (GAGs) (Hurst et al., 1987, Parsons et al., 1990) (see **Figure 1.9**). GAGs are extremely hydrophilic as the sulfate moieties of the polysaccharide chains readily bind water (Hurst et al., 1987, Parsons et al., 1990). It has been proposed that GAGs on the surface of the urothelium tightly bind a layer of water forming a mucus like layer which acts as a permeability barrier, preventing the absorption and adherence of small molecules, bacteria and proteins (Parsons et al., 1990, Parsons, 2007). It has been hypothesised that infection, interstitial cystitis, radiation and chemical cystitis may be due to loss or damage of this water/mucus layer (Parsons, 2007).

Figure 1.9 has been removed due to Copyright restrictions.

The original image can be viewed in Metts, J. F. 2001, 'Interstitial cystitis: urgency and frequency syndrome', Am Fam Physician, vol. 64, no. 7, pp. 1199-206 (refer to Figure 1).

Figure 1.9 Cross section of bladder wall and close up of bladder epithelial surface showing the uroplakin and GAG layers on the luminal surface of the urothelium (Metts, 2001).

Barrier Function

The barrier function of the bladder is dependent on several features of the umbrella cells including the tight junctions that reduce movement of ions, solutes and toxins between cells as well as the uroplakin molecules and GAGs which reduce permeability of the cells to small molecules such as water, urea and protons (Acharya et al., 2004, Apodaca, 2004). This barrier function must be maintained even during filling and emptying of the bladder. The increased volume of the bladder during the filling or storage stages is accommodated by the urothelium in at least two ways, the first being the unfolding of the highly wrinkled mucosal surface as well as a change in morphology of the urothelial cells (Apodaca, 2004). The urothelial cells become thinner as a result of intermediate and basal cells being pushed laterally as well as the umbrella cells changing shape from roughly cuboidal to flat and squamous (Apodaca, 2004). There is evidence that this shape change is accompanied by vesicular exocytosis adding membrane to the surface of the umbrella cell (Balestreire and Apodaca, 2007, Truschel et al., 2002, Wang et al., 2005).

Sensory & Secretory Function

However, the urothelium is not just a barrier as recent evidence suggests that it can respond to physical and chemical stimuli and communicate with underlying cells including nerves, interstitial cells and smooth muscle (Fry et al., 2007, Birder et al., 2010b). Additionally, the urothelial cells express a number of sensory receptors and ion channels which are similar to those found in sensory nerves (Andersson et al., 2010, Birder et al., 2010b). The sensory receptors/ions channels that have been found in urothelium include receptors for purines (P2X1–7 and P2Y1,2,4), adenosine (A1, A2a, A2b and A3) noradrenaline (α and β) and acetylcholine (muscarinic and nicotinic) among others (Burnstock, 2001, Birder et al., 2002b,

Chess-Williams, 2002, Beckel et al., 2006, Chopra et al., 2008). The expression of these receptors enables the urothelium to respond to sensory inputs such as ACh, ATP, adenosine or noradrenaline released from nearby nerves (Apodaca et al., 2007, Birder et al., 2010b). Furthermore, the urothelial cells themselves are capable of secreting transmitters or mediators such as ATP, ACh, peptides, prostaglandins, NO and cytokines that can modulate, activate or inhibit afferent nerves, smooth muscle, interstitial cells or even inflammatory cells (Downie and Karmazyn, 1984, Ferguson et al., 1997, Morrison, 1999, Namasivayam et al., 1999, Pinna et al., 2000, Apodaca et al., 2007, Birder and de Groat, 2007, Fry et al., 2007, Birder et al., 2010b). The sensory and secretory actions of urothelial cells are discussed in more detail below.

ATP

ATP can be released from cells by exocytosis, membrane transporters or anion-selective channels (Sabirov and Okada, 2004). ATP is released from urothelial cells in response to stretch during bladder filling (Ferguson et al., 1997, Vlaskovska et al., 2001). ATP released from the urothelium can act on P2X2 and P2X3 receptors on the urothelial cells in an autocrine manner to induce membrane exocytosis to accommodate a larger bladder volume (Wang et al., 2005). The presence of P2Y receptor subtypes has also been reported. Birder et al., (2004) demonstrated the presence of P2Y1, P2Y2 and P2Y4 subtypes in feline urothelium while Chopra et al., (2008) reported the presence of P2Y2 and P2Y4 in rat urothelial cells. Activation of the P2Y receptors in rat urothelial cells resulted in increased intracellular calcium and ATP release (Chopra et al., 2008).

The urothelial-derived ATP can also activate P2X3 receptors on sub-urothelial afferent nerves in a paracrine manner which then convey afferent information of bladder fullness to the central nervous system, leading to altered micturition reflexes (Cockayne et al., 2000, Burnstock, 2001, Vlaskovska et al., 2001). A study in P2X3 knockout mice demonstrated that a deficiency in the P2X3 receptor resulted in infrequent urination, increased bladder capacity, and failure to undergo bladder contractions when experimentally filled (Cockayne et al., 2000).

Nitric Oxide

Immunocytochemical studies in rats, cats and rabbits have revealed that urothelial cells contain NOS and that NO release can be stimulated by various chemicals and neurotransmitters including capsaicin, nicotine, acetylcholine, noradrenaline, CGRP and substance P (Birder et al., 1998, de Groat and Yoshimura, 2001, Birder et al., 2010b). It is believed that NO released from the urothelium may play a role in sensory mechanisms in the bladder (de Groat and Yoshimura, 2001). This is supported by studies showing that administration of an NO donor or L-arginine

reduces bladder reflex hyperactivity and reduces the pain and voiding frequency in interstitial cystitis (Smith et al., 1997, Ozawa et al., 1999). Specifically, rats with cyclophosphamide induced cystitis were given NO donors which increased the intercontraction interval without changing contraction amplitude indicating an inhibitory effect on bladder reflexes not the smooth muscle itself (Ozawa et al., 1999).

ACh

The urothelium releases ACh in response to stretch (Yoshida et al., 2006) which is thought to act in an autocrine fashion on muscarinic and nicotinic receptors on the urothelium (Wessler et al., 1998). It has also been shown that there is basal release of ACh from the human urothelium and that this may contribute to an auto-feedback mechanism to suppress its own release (Yoshida et al., 2004, Yoshida et al., 2006). Additionally, ACh from cholinergic nerves in the lamina propria adjacent to the urothelium may activate receptors on the urothelium (Birder et al., 2010b).

The urothelium of several species has been shown to express muscarinic receptors including rat, pig and human (Tyagi et al., 2006, Giglio et al., 2005, Hawthorn et al., 2000). In the human urothelium all muscarinic receptor subtypes have been found (M1-M5) with the M1 subtype reported on basal cells, M2 on umbrella cells, M3 and M4 throughout the urothelium and M5 in a decreasing gradient from luminal to basal cells (Bschleipfer et al., 2007). The activation of muscarinic receptors in the urothelium leads to release of several transmitters including ATP, NO and PGE₂ (Birder et al., 1998, Kullmann et al., 2008, Giglio and Tobin, 2009, Yokoyama et al., 2011, Nile and Gillespie, 2012).

The release of ACh may also affect afferent nerve activity in the bladder. The effect of muscarinic receptor stimulation on afferent nerve activity is still unclear however several studies report that intravesical and systemic administration of muscarinic antagonists decreased afferent nerve activity and increased micturition interval and bladder capacity (Yokoyama et al., 2005, Hedlund et al., 2007, Iijima et al., 2007). However, another study has reported that antagonism of muscarinic receptors had no effect on afferent nerve activity and rather stimulation of muscarinic receptors inhibited afferent responses to distension (Daly et al., 2010). Accordingly, the role of ACh released from urothelium is not yet clear and further investigation is required.

Nicotinic $\alpha 7$ - and $\alpha 3$ -containing receptors have also been shown to be present in the urothelium and their activation leads to inhibition and excitation of bladder voiding reflexes respectively (Beckel and Birder, 2012). These effects appeared to be due to regulation of mediator release

from the urothelium. Specifically, the excitatory response to $\alpha 3$ receptor stimulation could be blocked by the purinergic antagonists PPADS suggesting nicotinic receptor regulation of ATP release which then enhances afferent activity (Beckel and Birder, 2012).

Urothelium Derived Inhibitory Factor

Muscarinic receptor stimulation also causes the urothelium to release an unidentified factor that inhibits contraction of the underlying smooth muscle called the urothelium derived inhibitory factor (UDIF) (Hawthorn et al., 2000, Chaiyaprasithi et al., 2003). Removal of the urothelium resulted in a significant increase in the detrusor muscle contraction in response to ACh (Hawthorn et al., 2000, Chaiyaprasithi et al., 2003). The UDIF has been investigated in various species including pig and human and is neither NO, noradrenaline, a COX product, a catecholamine, adenosine, gamma-aminobutyric acid nor an endothelium-derived hyperpolarising factor sensitive to apamin (Hawthorn et al., 2000, Chaiyaprasithi et al., 2003).

Noradrenaline

Excitatory β -AR are also present in the urothelium and stimulation of these receptors leads to NO release (Birder et al., 2002b). As discussed above, NO released from the urothelium is believed to have an inhibitory effect on bladder reflexes leading to increased intercontraction interval and decreased voiding frequency (Smith et al., 1997, Ozawa et al., 1999).

Prostaglandins

The urothelium expresses COX-1 and COX-2 enzymes which are responsible for the synthesis of prostanoids including prostaglandins (de Jongh et al., 2009). Prostaglandins released from the urothelium are thought to activate or sensitise afferent nerves (de Groat and Yoshimura, 2001). In addition, PGE₂, released from afferent nerves or the urothelial cells and acting via EP1 and/or EP3 receptors on urothelial cells stimulates the release of ATP (Tanaka et al., 2011).

TRP Channels

Recent evidence has suggested that TRP channels can be found on urothelial cells not just on sensory neurones in the bladder of mice and humans (Everaerts et al., 2010a, Apostolidis et al., 2005, Lazzeri et al., 2005, Lazzeri et al., 2004, Birder et al., 2002a). Yu et al., (2011) demonstrated, using Western blotting, the urothelial expression of TRPC1, TRPC4, TRPV1, TRPV2, TRPV4, TRPM4, TRPM7 and TRPML1 proteins. Specifically, they found that TRPV2 and TRPM4 were prominently localised to the umbrella cell apical membrane, while TRPC4 and TRPV4 were identified on their abluminal surfaces and TRPC1, TRPM7, and TRPML1 were localised to the cytoplasm (Yu et al., 2011). This study did not identify whether these receptors were functional. However, TRP channels are activated by various physical stimuli, such as heat

and stretch, as well as chemical stimuli such as low pH, osmolality and noxious stimuli and are therefore thought to act as sensors of stretch or chemical irritation in the bladder (Everaerts et al., 2008, Tominaga et al., 1998, Tominaga and Tominaga, 2005).

A study using wild type and TRPV1 knockout mice indicated that activation of TRPV1 channels on urothelial cells may cause NO release (Birder et al., 2002a). Furthermore, the TRPV1 knockout mice had a lower level of stretch evoked ATP release indicating a role for TRPV1 channels in purinergic signalling by the urothelium (Birder et al., 2002a). The TRPV1 (-/-) mice had a higher frequency of low-amplitude, non-voiding bladder contractions accompanied by decreased spinal cord signalling and reflex voiding during bladder filling suggesting a role in urine storage (Birder et al., 2002a). Another study using cultured mouse urothelial cells demonstrated the presence of functional TRPV2, 4 and 7 channels (Everaerts et al., 2010a). Functional TRPV4 channels, which can be activated by hypo-osmolality, heat or certain lipid compounds, have been found in rat and mouse urothelium (Gevaert et al., 2007) and TRPV4-null mice demonstrated a lower frequency of voiding and higher frequency of nonvoiding contractions (Gevaert et al., 2007). Another study found that activation of TRPV4 channels resulted in the release of ATP and increased detrusor muscle contraction after micturition (Birder et al., 2007).

The role of the TRP channels in bladder function is still not well understood, however from the evidence these channels appear to be involved in the sensory mechanisms of the urothelium.

Epithelial Sodium Channels

Epithelial Sodium Channels (ENaC) are cation channels characterised by amiloride-sensitivity, and can be activated by mechanical stimuli and/or by ligands such as peptides or protons (Araki et al., 2008). ENaC have been identified in the urothelium (Araki et al., 2004, Smith et al., 1998) and have been shown to be mechanosensitive, changing sodium transport properties in response to hydrostatic pressure changes (Ferguson et al., 1997). It has also been suggested that ENaCs are involved in mechanosensory transduction by modulating stretch-evoked ATP release. For example, the basal ATP release from the rabbit bladder epithelium is altered by amiloride, a blocker of ENaC (Ferguson et al., 1997). In cultured cat urothelial cells amiloride significantly reduced the ATP released by hypotonic stimulus (Birder et al., 2003). In rats, amiloride has also been shown to inhibit ATP release from urothelial cells in response to stretch (Du et al., 2007a) and hydrostatic pressure (Olsen et al., 2011). Furthermore, ENaC were found to be over expressed in the urothelium of patients with bladder outlet obstruction potentially indicating a role for these channels in the detrusor overactivity experienced by these

patients (Araki et al., 2004). Therefore, it appears that ENaCs may also be involved in the mechanosensory mechanisms in the bladder.

Peptides

As mentioned above, bladder afferent neurones contain various neuropeptides that can be released as a result of noxious stimulation of the nerves (de Groat and Yoshimura, 2001, Keast and De Groat, 1992, Morrison, 1999). Substance P can act on NK receptors on the urothelium to cause release of ATP and NO (Munoz et al., 2010, de Groat and Yoshimura, 2001). Neurokinin A and neurokinin B have been demonstrated to activate NK receptors, specifically the NK2 subtype, in the urothelium in normal unanaesthetised rats resulting in micturition, however the mechanism of this action is unclear (Ishizuka et al., 1995a).

Nerve growth factor

Nerve growth factor (NGF) is produced in the urothelium and its expression is increased in bladder dysfunction such as interstitial cystitis (Liang et al., 2010, Lowe et al., 1997, Ochodnický et al., 2011). It is thought that NGF may be released from the urothelium and may act in a paracrine or autocrine manner. Intravesical application of NGF induces rapid and enduring sensitisation of A- δ and C afferent fibres to mechanical stimuli in rats which results in bladder overactivity, reduced micturition volume and increased number of bladder contractions (Dmitrieva and McMahon, 1996). NGF binds to tyrosine kinase receptors (Trks) and the TrkA and TrkB subtypes have been found in the normal urothelium (Murray et al., 2004). The role of these receptors in the urothelium is not known.

Relevance of the Urothelium

As the urotoxic metabolites of CPO and IFO come into direct contact with the urothelium, it is this layer which is of particular interest in this project. Understanding what changes are occurring in this layer and how they affect the function of the bladder as whole may lead to identification of possible targets for preventing or alleviating the adverse effects of these drugs, potentially leading to increased drug toleration and better treatment outcomes.

Table 1.2: Summary of receptor expression in the urothelium

Receptor / stimulus	Subtype present	Species	Reference
Muscarinic	M1-M5	Rat	(Giglio et al., 2005)
		Human	(Bschleipfer et al., 2007, Mansfield et al., 2005, Tyagi et al., 2006)
Nicotinic	$\alpha 3$, $\alpha 7$, $\alpha 5$, $\beta 3$ -4	Rat	(Beckel et al., 2006)
	$\alpha 7$, $\alpha 9$, $\alpha 10$	Human	(Bschleipfer et al., 2007)
Adrenergic	β -AR	Rat	(Birder et al., 2002b)
	β -AR	Human	(Harmon et al., 2005)
Purinergic	P2X5, P2X6, P2X7	Rat	(Lee et al., 2000)
	P2X3	Rat & human	(Elneil et al., 2001)
	P2X1-7 P2Y1, 2, 4	Cat	(Birder et al., 2004)
	P2Y2, P2Y4	Rat	(Chopra et al., 2008)
Prostaglandin E ₂	EP1 / EP3	Rat	(Tanaka et al., 2011)
TRP Channels	TRPC1, TRPC4, TRPV1, TRPV2, TRPV4, TRPM4, TRPM7, TRPML1	Mouse	(Yu et al., 2011)
	TRPV1	Mouse	(Birder et al., 2002a)
	TRPV4	Rat & mouse	(Gevaert et al., 2007, Birder et al., 2007)
	TRPV2, 4, 7	Mouse	(Everaerts et al., 2010a)
	TRPA1	Rat	(Streng et al., 2008)
Neurokinin	NK2	Rat	(Ishizuka et al., 1995a)
Nerve Growth Factor	TrkA and TrkB	Rat	(Murray et al., 2004)
Amiloride sensitive Na ⁺ channel	ENaC	Rat & rabbit	(Watanabe et al., 1999, Burton et al., 2002)
		Human	(Araki et al., 2004)

Interstitial cells

Cells resembling the interstitial cells of Cajal of the gastrointestinal tract have been identified in the bladder (Smet et al., 1996). These cells have been given various names in the literature but are referred to throughout this thesis as simply interstitial cells (IC). Two populations of IC have been identified, one in the lamina propria referred to as IC-LP and the second in the detrusor muscle referred to as intramuscular or IC-IM (McCloskey, 2010, Davidson and McCloskey, 2005). The IC-LP are stellate shaped with branches showing interaction with nearby nerves in the lamina propria. The IC-LP express the gap junction protein connexin-43 which allows cells to communicate with each other, potentially forming an electrical network (Ikeda et al., 2007, Sui et al., 2002). In the detrusor two types of IC-IM were identified (Davidson and McCloskey, 2005). The first type were characterised by an elongated cell morphology and were not interconnected but were found running in parallel with smooth muscle bundles and in close connection with intramural nerves. The second type had stellate cell morphology and were located in the interstitial space between muscle bundles and formed isolated areas of interconnected cells (Davidson and McCloskey, 2005).

There has been much debate about whether IC are involved in the initiation of spontaneous activity of the bladder smooth muscle (Brading and McCloskey, 2005). In patients with overactive bladder increased IC-LP have been reported suggesting that their upregulation is linked to the increased spontaneous contractions experienced by patients during bladder filling (Roosen et al., 2009). The IC have calcium-activated chloride channels which are necessary for a pacemaking role (Wu et al., 2004). The IC-IM also exhibit spontaneous Ca^{2+} fluctuations which were suggested to mediate the spontaneous activity of the detrusor smooth muscle (Hashitani et al., 2004, Johnston et al., 2008). However, electrophysiological measurements of isolated IC demonstrated that the fluctuations in membrane potential do not reach a level sufficient to cause an action potential (0mV) and therefore are unlikely to act as pacemakers (Sui et al., 2004). Rather, it was suggested that a group of cells stimulated simultaneously could spread or amplify an excitatory effect.

However, there is growing evidence that the IC-LP may be involved in the sensory response to bladder wall changes mediating signals between the urothelium and the afferent nerves or detrusor muscle (Brading and McCloskey, 2005, Fry et al., 2007, Sui et al., 2008). The urothelium responds to bladder filling by releasing chemical mediators such as ATP, ACh and PGE_2 (Birder et al., 2010b). The ICs express various receptors for these agonists which suggests they may be able to sense and respond to inputs from the urothelium. Wu et al.,

(2004) showed that isolated guinea-pig IC-LP responded to exogenous ATP by generating an intracellular Ca^{2+} transient followed by inward currents and this response was concentration-dependent. Similar transients were generated by UTP and ADP but not by $\alpha\beta\text{mATP}$ suggesting that ATP acts via a P2Y receptor. Tissue sections and isolated cells from guinea-pig bladder showed strong labelling with the P2Y6 antibody and weak labelling for P2X3, P2Y2 and P2Y4 (Sui et al., 2006). Immunoreactivity for M2 and M3 receptors has also been reported in cells resembling IC in the lamina propria (Mukerji et al., 2006b, Grol et al., 2009). However, conflicting evidence exist about whether IC respond to muscarinic stimulation (Johnston et al., 2008, Lagou et al., 2006, McCloskey and Gurney, 2002, Sui et al., 2004). In addition, IC expression of PGE_2 receptors EP1 and EP2 has been demonstrated suggesting they may be able to respond to PGE_2 release by the urothelium (Rahnama'i et al., 2010). Therefore, current research indicates a regulatory or facilitatory role for bladder ICs in transducing the urothelial sensory information to nerves and detrusor.

1.4 CYCLOPHOSPHAMIDE AND IFOSFAMIDE

Cyclophosphamide (CPO) and ifosfamide (or ifosfamide) (IFO) are commonly used to treat a variety of malignant and non-malignant neoplastic diseases including malignant lymphomas, leukaemias, neuroblastoma, retinoblastoma and carcinomas of the ovary, breast, endometrium and lung, advanced stage solid tumours, as well as being used as immunosuppressive agents in bone marrow transplantation and chronic autoimmune disorders such as rheumatoid arthritis, autoimmune skin disease, multiple sclerosis, systemic vasculitides and systemic lupus erythematosus (Sladek, 1988, Nichols, 1995, Bergh, 2003, Buda et al., 2003, de Jonge et al., 2005, Lawson et al., 2008).

The dose, timing and route of CPO and IFO administration vary with age of the patient and the condition being treated. However, the reported doses of CPO range from 50-1000mg/m² per oral (p.o.) or 500 – 6000 mg/m² intravenously (i.v.) over varying times of 1-96 hours (for review see (de Jonge et al., 2005)). Reported doses of IFO range from 1000-2000mg/m² p.o. or 1000mg-18g/m² i.v. over 1-120 hours (for review see (Kerbusch et al., 2001a)). A more detailed discussion of the doses of CPO and IFO given and the relevant urinary concentrations can be found later in this Chapter.

Structure

The structure of cyclophosphamide 2-[bis(2-chloroethyl)amino]tetrahydro-2H-1,3,2-oxazaphosphorine 2-oxide is characterised by a nitrogen-mustard group (bis-chloroethylamine) attached to an oxazaphosphorine ring (Cox, 1979, de Jonge et al., 2005). Ifosfamide [3-(2-chloroethyl)-2-(2-chloroethylamino)-2-oxide] is a structural isomer of cyclophosphamide, the only difference between the two being that the chloroethyl functions are located on different nitrogen atoms (Cox, 1979, Wagner, 1994). **Figure 1.10** shows the structural formulas of cyclophosphamide and ifosfamide.

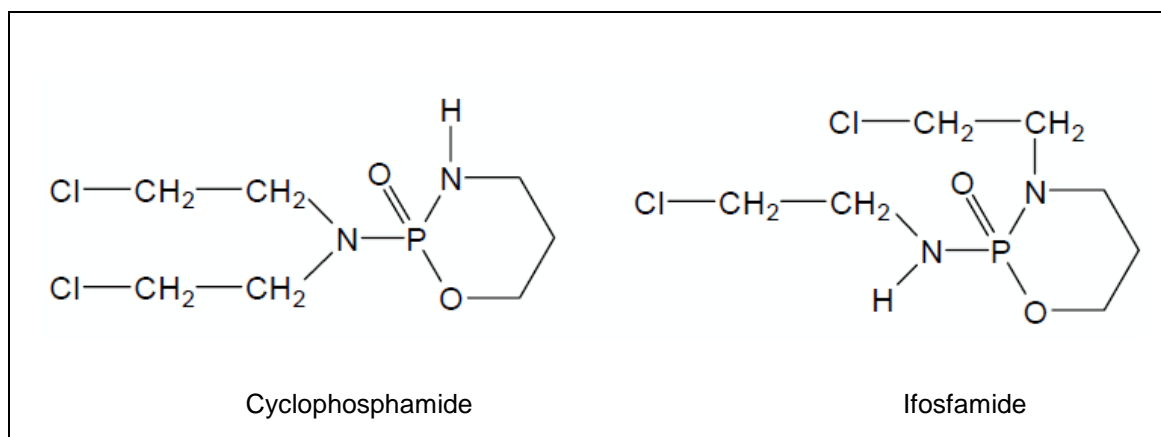


Figure 1.10 Cyclophosphamide and ifosfamide structural formulas.

Both CPO and IFO are inactive pro-drugs that must be activated by enzymes, primarily in the liver, to produce the active drug (Furlanut and Franceschi, 2003, de Jonge et al., 2005). CPO and IFO are metabolised to produce the active alkylating or cytotoxic agent which is phosphoramidate mustard or ifosforamide mustard respectively (Connors et al., 1974, Struck et al., 1975, Colvin et al., 1976, Sladek, 1988, Kerbusch et al., 2001a, Furlanut and Franceschi, 2003, de Jonge et al., 2005). Ifosforamide mustard is an analogue of the phosphoramidate mustard of cyclophosphamide (Kerbusch et al., 2001a). **Figure 1.11** shows the structural formulas of phosphoramidate mustard and ifosforamide mustard.

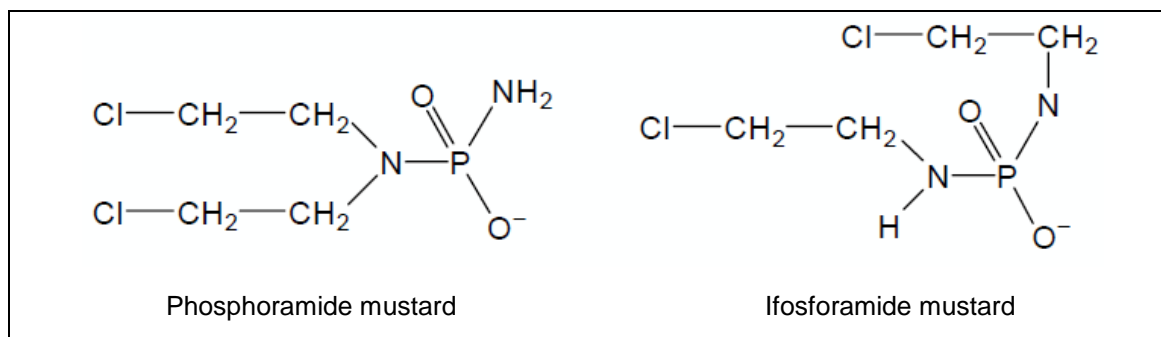


Figure 1.11 Phosphoramidate mustard and ifosforamide mustard structural formulas.

Metabolism

Although the structural formula of each drug is different the metabolic pathway is similar and both drugs are extensively metabolised into active and inactive metabolites (Sladek, 1988, Wagner, 1994, Furlanut and Franceschi, 2003). The metabolism of both CPO and IFO is broken into the activation pathway and the deactivation pathway. **Error! Reference source not found.** and **Figure 1.13** show the metabolism pathway of cyclophosphamide and ifosfamide respectively.

Figure 1.12 has been removed due to Copyright restrictions.

The original image can be viewed in Boddy, A. V. & Yule, S. M. 2000, 'Metabolism and pharmacokinetics of oxazaphosphorines', Clin Pharmacokinet, vol. 38, no. 4, pp. 291-304. (refer to Figure 1).

Figure 1.12 Metabolism of cyclophosphamide (Boddy and Yule, 2000).

Figure 1.13 has been removed due to Copyright restrictions.

The original image can be viewed in Boddy, A. V. & Yule, S. M. 2000, 'Metabolism and pharmacokinetics of oxazaphosphorines', Clin Pharmacokinet, vol. 38, no. 4, pp. 291-304. (refer to Figure 2).

Figure 1.13 Metabolism of ifosfamide (Boddy and Yule, 2000).

Activation

Cyclophosphamide and ifosfamide are activated by the hydroxylation of the oxazaphosphorine ring at the carbon-4 position to form 4-hydroxycyclophosphamide and 4-hydroxyifosfamide in equilibrium with their ring-open tautomer aldophosphamide and aldoifosfamide respectively (Connors et al., 1974, Friedman et al., 1976, Fenselau et al., 1977, Furlanut and Franceschi, 2003, de Jonge et al., 2005). Both CPO and IFO are activated primarily in the liver by hepatic microsomal mixed-function oxidases (cytochrome P450 [CYP] enzyme system) (Connors et al., 1974, Fenselau et al., 1977, de Jonge et al., 2005). Numerous CYP isoenzymes are involved in the bioactivation of CPO and IFO in humans including CYP2A6, 2B6, 3A4, 3A5, 2C9, 2C18 and 2C19 (Chang et al., 1993, Chang et al., 1997, de Jonge et al., 2005, Griskevicius et al., 2003, Huang et al., 2000, Ren et al., 1997, Roy et al., 1999, Xie et al., 2003, Yu and Waxman, 1996). Analysis of CYP enzyme expression in human liver suggests that CYP2B6 has the greatest involvement in CPO activation while CYP3A3 and 3A4 appear to have the highest involvement in IFO activation (Chang et al., 1993, Roy et al., 1999, Xie et al., 2003).

Of an administered dose of cyclophosphamide, approximately 70-80% is activated in the liver and the remainder is either inactivated by side-chain oxidation to dechloroethylcyclophosphamide and chloroacetaldehyde or is excreted unchanged in the urine,

bile, breath and faeces (Bagley et al., 1973, Dooley et al., 1982, Bailey et al., 1991, Joqueviel et al., 1998, Busse et al., 1999, Boddy and Yule, 2000, de Jonge et al., 2005). The activation of IFO is similar to that of CPO, however closer to 50% of a dose of ifosfamide will be activated to 4-hydroxyifosfamide with the remainder inactivated via side-chain oxidation or excreted unchanged (Sladek, 1988, Boddy and Yule, 2000, Kerbusch et al., 2001a).

4-Hydroxycyclophosphamide/aldophosphamide and 4-hydroxyifosfamide/aldoifosfamide are not cytotoxic themselves but readily diffuse into cells where they spontaneously form phosphoramidate mustard and ifosforamide mustard respectively by β -elimination of acrolein (Alarcon and Meienhofer, 1971, Friedman et al., 1976, Connors et al., 1974, Boyd et al., 1986, Sladek, 1988, Busse et al., 1999, Kerbusch et al., 2001a, de Jonge et al., 2005, Misiura, 2006). The decomposition of 4-hydroxycyclophosphamide/aldophosphamide and 4-hydroxyifosfamide/aldoifosfamide may be partly catalysed by albumin or other proteins (Hohorst et al., 1986, Kwon et al., 1987, Sladek, 1988).

Deactivation

In addition to the activation pathway discussed above, a number of inactive metabolites are also produced during metabolism of CPO and IFO. One of the inactive metabolites of CPO is dechloroethylcyclophosphamide which is produced by an oxidative *N*-dealkylation reaction (de Jonge et al., 2005) producing an equimolar amount of chloroacetaldehyde (CAA) (Connors et al., 1974, Bohnenstengel et al., 1996, Yu and Waxman, 1996, Ren et al., 1997, Huang et al., 2000). The intermediate 4-hydroxycyclophosphamide may be deactivated to ketophosphamide by an alcohol dehydrogenase (Sladek, 1988, Joqueviel et al., 1998, Busse et al., 1999). While, aldophosphamide can be deactivated by oxidation to carboxycyclophosphamide by an aldehyde dehydrogenase (Sladek, 1988, Dockham et al., 1992, Joqueviel et al., 1998, Busse et al., 1999). The production of carboxyphosphamide from aldophosphamide is the most important detoxification pathway of cyclophosphamide (de Jonge et al., 2005).

The primary deactivation pathway for ifosfamide involves the removal of a chloroethyl group from either the exo- or endocyclic nitrogen atom to form nontoxic 2-dechloroethylifosfamide and 3-dechloroethylifosfamide respectively as well as an equimolar amount of CAA (Kerbusch et al., 2001a). Dechloroethylation accounts for 25 to 60% of the metabolism of ifosfamide (Misiura et al., 1983, Sladek, 1988, Kaijser et al., 1994, Kerbusch et al., 2001a). The intermediate 4-hydroxyifosfamide can be inactivated to 4-ketoifosfamide and 4-thioifosfamide while the detoxification of aldoifosfamide yields both carboxyifosfamide and alcoifosfamide (Kerbusch et al., 2001a).

Detoxification of phosphoramidate mustard, ifosfamide mustard, acrolein and CAA occurs via conjugation with intracellular glutathione (GSH) either spontaneously or mediated by the enzyme glutathione-S-transferase (Gurtoo et al., 1981, Dirven et al., 1994, Gamcsik et al., 1999, Sood and O'Brien, 1993).

Mode of Action

Intracellular activation of CPO and IFO to phosphoramidate mustard and ifosfamide mustard results in the conversion of both 2-chloroethyl groups of each molecule into reactive electrophilic alkyl groups ($R-CH_2^+$) which in turn react with the nucleophilic moieties of DNA bases such as N^7 -guanine (Kerbusch et al., 2001a, de Jonge et al., 2005). The bifunctional character of phosphoramidate mustard and ifosfamide mustard derives from their ability to form two reactive alkyl groups (Kerbusch et al., 2001a). Specifically, phosphoramidate mustard has two exocyclic 2-chloroethyl groups while ifosfamide mustard has one 2-chloroethyl group on the exo and one of the endocyclic oxazaphosphorine nitrogen atom (Brock, 1983, Kerbusch et al., 2001a).

The two reactive alkyl groups can attach to two bases in the same DNA strand to form an intra-strand link or can attach to one base on two different DNA strands forming an inter-strand link. Inter-strand links, in particular, impair DNA replication by inhibiting double-strand separation prior to cell division leading to cell death by apoptosis (O'Connor et al., 1991, Povirk and Shuker, 1994, Dong et al., 1995, Shulman-Roskes et al., 1998, de Jonge et al., 2005). Therefore, since tumour cells have a high rate of cellular divisions, they will be affected by CPO and IFO at a greater rate than normal cells (Kerbusch et al., 2001a, de Jonge et al., 2005).

In addition, the metabolites acrolein and CAA may enhance the cytotoxicity of phosphoramidate mustard and ifosfamide mustard by depletion of intracellular GSH (Lind et al., 1989, Bruggemann et al., 1997, Borner et al., 2000, Gurtoo et al., 1981, Crook et al., 1986a, Crook et al., 1986b).

Elimination

Most of the literature reports urinary excretion of CPO, IFO and their metabolites as a percentage of the initial dose and very little information is available concerning the actual urinary concentrations of these substances as found in patients. In addition, there is large inter-individual variability seen in the urinary excretion of CPO and IFO. This makes it difficult to define a clinically relevant concentration of CPO, IFO or their metabolites to use in this study.

However, several studies have shown that the urinary elimination of CPO and IFO and its metabolites is almost complete 24 hr after the start of treatment (Bagley et al., 1973, Jardine et al., 1978, Sladek et al., 1980, Joqueviel et al., 1997). Specifically, Joqueviel et al., (1998) analysed urine samples from patients treated with CPO on two consecutive days and measured CPO as well its metabolites and their degradation products. They found that only 6% of the injected dose on day one and 3% on day two were recovered in the urine samples collected 18-24 hours after the beginning of CPO infusion and saw little interpatient variability. Furthermore, approximately 15% of the dose of CPO was excreted unchanged while carboxycyclophosphamide and its two degradation products were found to be the major metabolites of CPO (11.5% and 23% after the first and second doses, respectively) (Joqueviel et al., 1998).

Similar excretion results have been reported for IFO. In a study of 11 patients who received IFO in a number of treatment cycles with bolus doses or intravenous infusions (Singer et al., 1998). The study reported that approximately 23% of the administered dose of IFO was excreted in urine unchanged while 5.4% was excreted as the metabolite 2-dechloroethylifosfamide, 12.3% as 3-dechloroethylifosfamide, 14.8% as carboxyifosfamide and 9.9% as ifosforamide mustard.

Dechloroethylcyclophosphamide and CAA are primarily eliminated in the urine and account for <5% of total elimination of cyclophosphamide (Boddy et al., 1992, Joqueviel et al., 1998, Busse et al., 1999). However, the dechloroethylation pathway accounts for 25-60% of IFO metabolism in adults (Misiura et al., 1983, Sladek, 1988, Kaijser et al., 1994, Kerbusch et al., 2001b).

Another study measured urinary acrolein excretion in 16 bone marrow transfer patients receiving cyclophosphamide wherein urine samples were taken over a 24 hour period (Al-Rawithi et al., 1998). The amount of acrolein excreted in 24 hours was between 0.47% and 6.1% of administered cyclophosphamide with a mean of $1.96\% \pm 0.35$. The variation between patients was investigated and no correlation between dose, hydration status, previous chemotherapy or concomitant medication was found and was contributed to the high interpatient variability in metabolising CPO (Al-Rawithi et al., 1998).

Urinary Concentrations

For the purpose of this review, it is important to estimate the clinically relevant urinary concentrations of CPO, IFO and their major metabolites acrolein and CAA as it is the urinary concentration that is in contact with the urothelium that must be replicated *in vitro*. One study of

16 breast cancer patients receiving intravenous CPO at a dose of 600 or 1200 mg/m² reported actual urinary concentrations of CPO to be approximately 10-400µM at approximately 20-24 hours after administration of CPO (Hedmer et al., 2008). Unfortunately, this study did not measure plasma or urine concentrations of CPO before 20 hours.

For the parent drug IFO, no reliable urinary concentration data could be found in the literature. However, in a study by Kurowski & Wagner (1993), the plasma concentrations of IFO and its metabolites were investigated in 11 patients with bronchogenic carcinoma receiving intravenous IFO on a 5-day divided-dose schedule (1.5 g/m² daily). Peak plasma concentrations of IFO on day 1 and 5 were approximately 200µM.

For acrolein, one study measured the urinary concentrations in 19 patients between the ages of 1 year and 21 years receiving varying intravenous doses of CPO and IFO for different diseases. Urinary acrolein concentrations peaked at times between 1 and 12 h after starting therapy (mean±S.D., 5.0±2.7) and maximum urinary acrolein concentrations ranged from 0.3 to 406.8nM (mean± S.D., 39.7±76.7 nM) (Takamoto et al., 2004). The measurement of 406.8nM was an outlier in the data with the next highest peak urine concentration being 111.3nM.

Kurowski & Wagner (1993) also measured CAA and found plasma concentrations on day 1 and 5 reached a maximum of 2-5µM respectively. In another study, blood samples from one patient treated with a ten-day continuous infusion of IFO revealed plasma concentrations of CAA that did not exceed 10µM (Kaijser et al., 1993). Furthermore, Pendyala et al., (2000) measured various metabolites in 24 patients receiving intravenous IFO ranging from 2 to 8g/m² and found that the maximum plasma CAA concentrations were 10–35µM. The only study found to report actual urinary concentrations in six children receiving 1.6g/m² ifosfamide intravenously every day for 5 days reported concentrations up to 221µM while average plasma concentrations in the same patients ranged from 22µM-109µM (Goren et al., 1986). Peak concentrations of CAA were also seen within 24 hours.

Accordingly, the range of concentrations deemed to be clinically relevant for CPO, IFO, acrolein and CAA was 10nM – 100µM. This range was tested initially and then toxic concentrations used in subsequent experiments.

Urotoxicity - Mechanisms, Effects & Protection

The urotoxic effects of CPO or IFO can be split into anatomical and functional effects. The metabolites of CPO and IFO come into direct contact with the urothelium causing damage and resulting in inflammation. This inflammation causes oedema, ulceration, neovascularisation,

haemorrhage and necrosis or apoptosis (Cox, 1979, Lima et al., 2007). Whereas, the functional effects can manifest as bladder pain, urinary frequency, urgency, dysuria and feelings of incomplete emptying (Korkmaz et al., 2007, Fukuoka et al., 1991) and these effects can last well beyond the resolution of inflammation and damage.

Mechanism of toxicity

Almost exclusively, the toxic effects of CPO and IFO have been attributed to acrolein damaging and infiltrating the urothelium and initiating an inflammatory response. Mice treated with CPO showed intense protein-acrolein adduct formation localised to the lamina propria accompanied by the most intense focal damage including oedema, haemorrhage, schistocyte formation, albumin extravasation, ulcerative dissolution of connective tissue, inflammatory cell infiltration, and apoptosis (Conklin et al., 2009).

Acrolein is a highly electrophilic, reactive α,β -unsaturated aldehyde which will rapidly bind to and deplete nucleophilic cell constituents, such as GSH. Acrolein can also bind to lysine, histidine and cysteine residues of proteins and nucleophilic sites in DNA resulting in widespread protein and DNA modification (Beauchamp et al., 1985, Kehrer and Biswal, 2000, Korkmaz et al., 2007). In high concentrations acrolein is cytotoxic and can lead to necrosis or apoptosis of cells (Beauchamp et al., 1985, Li et al., 1997). At lower concentrations acrolein can react rapidly at many cellular sites including depleting or binding cellular thiols and proteins, activating lipid peroxidation (Adams and Klaidman, 1993) and activating genes, either directly or subsequent to effects of transcription factors such as nuclear factor-kappaB (NF- κ B) (Horton et al., 1999) and activator protein-1 (AP-1) (Biswal et al., 2002, Korkmaz et al., 2007). Acrolein has also been shown to cause single-stranded DNA breaks (Erickson et al., 1980).

It has been suggested that the main mechanism by which acrolein causes bladder damage is through the production of reactive oxygen species (ROS) and NO (Korkmaz et al., 2007). The inhibition of NO synthesis leads to a reduction of both oedema and haemorrhage caused by CPO treatment of rats (Souza-Fiho et al., 1997). Acrolein rapidly enters urothelial cells and activates intracellular ROS and NO production either directly or via various mediators including NF- κ B and AP-1 ultimately producing peroxynitrite (ONOO⁻). The increased level of ONOO⁻ results in lipid peroxidation, protein oxidation and DNA damage leading to poly ADP-ribose polymerase (PARP) activation (Korkmaz et al., 2007). PARP is a DNA repair enzyme that can become over activated when DNA is damaged which can lead to the depletion of oxidised nicotinamide-adenine dinucleotide (NAD) and ATP, and consequently in apoptotic or necrotic cell death (Korkmaz et al., 2007). **Figure 1.14** shows this proposed mechanism in more detail.

Figure 1.14 has been removed due to Copyright restrictions.

The original image can be viewed in Korkmaz, A., Topal, T. & Oter, S. 2007, 'Pathophysiological aspects of cyclophosphamide and ifosfamide induced hemorrhagic cystitis; implication of reactive oxygen and nitrogen species as well as PARP activation', Cell biology and toxicology, vol. 23, no. 5, pp. 303-12. (refer to Figure 2).

Figure 1.14: Proposed mechanism of acrolein urotoxicity. (I) Acrolein enters urothelial cell and causes ROS production, iNOS induction, and activation of transcription factors (e.g. NF- κ B and AP-1). Activated transcription factors cause cytokine (TNF- α , IL-1 β) gene expression, iNOS induction and further ROS production. (II–III) Cytokines diffuse out into other urothelial cells, smooth muscle cells and into the bloodstream. ROS and NO form peroxynitrite (ONOO-) in urothelium and detrusor, leading to lipid peroxidation, protein oxidation, and DNA damage. DNA damage leads to PARP activation, depletion of NAD and ATP and eventually cellular necrosis. (IV) During necrosis, harmful intracellular contents are released into surrounding tissue potentially damaging neighbouring cells (Korkmaz et al., 2007).

Acrolein is not only a urotoxic metabolite of CPO and IFO but is an environmental pollutant which humans are exposed to in many common situations including as part of cigarette smoke, forest and house fires and part of automotive exhaust (Tanel and Averill-Bates, 2007). Acrolein has been implicated in chronic obstructive pulmonary disease (Borchers et al., 1999) as well as neurodegenerative disorders such as Alzheimer's disease (Lovell et al., 2001). Acrolein toxicity has been demonstrated in a number of tissues other than bladder and induces apoptosis in some tissues such as keratinocytes (Takeuchi et al., 2001), bronchial epithelial cells (Nardini et al., 2002), cultured neurons (Pugazhenthir et al., 2006), and Chinese hamster ovary cells (Tanel and Averill-Bates, 2005). However, studies in other tissues have reported acrolein induced necrotic cell death including in neutrophils (Finkelstein et al., 2005) as well as cultured neuronal cells (Liu-Snyder et al., 2006, Luo et al., 2005). However, several of these studies reported cell death by apoptosis at low concentrations of acrolein and necrotic cell death at higher

concentrations which may also explain these variable findings (Finkelstein et al., 2005, Tanel and Averill-Bates, 2005).

Acrolein induced apoptosis appears to be due to activation of the intrinsic/mitochondrial pathway which involves the activation of various caspases and altered activation of various transcription factors and promoters that are involved in apoptosis and cell survival (Tanel and Averill-Bates, 2005). Altered activation of these factors ultimately leads to chromatin condensation, cytoskeletal changes, nuclear membrane breakage, DNA fragmentation, cell blebbing, and formation of apoptotic bodies which are then phagocytosed by macrophages (Tanel and Averill-Bates, 2005). Acrolein-induced apoptosis in cultured bronchial epithelial cells was also associated with depletion of cellular GSH and intracellular generation of oxidants (Nardini et al., 2002). Another study in cultured neuroblastoma cells treated with acrolein demonstrated that acrolein induced the production of ROS, nicotinamide-adenine dinucleotide phosphate-oxidase activity, depletion of GSH, protein oxidation/nitration and lipid peroxidation (Huang et al., 2000).

However another toxic metabolite excreted in the urine is chloroacetaldehyde (CAA) which is produced during detoxification of cyclophosphamide and ifosfamide (Shaw et al., 1983). CAA has been suggested to play a role in the cardio- neuro- and nephro-toxicity of CPO and IFO (Goren et al., 1986, Sladek, 1988, Joqueviel et al., 1997) however no data has reported its role, if any, in urotoxicity. CAA is also highly electrophilic and toxicity occurs by similar mechanisms to acrolein including depletion of cellular reduced GSH, NAD and ATP, disturbed Ca^{2+} signalling, and lipid peroxidation, potentially leading to cell death via apoptosis or necrosis (Nissim et al., 2006, Schwerdt et al., 2006, Sood and O'Brien, 1993). This evidence suggests that CAA may be involved in the urotoxicity of CPO and IFO and accordingly CAA urotoxicity was investigated in the following studies.

Effects of urotoxicity

As mentioned above, CPO or IFO treatment can cause anatomical and functional effects.

Anatomical effects

Anatomical effects such as oedema, ulceration, neovascularisation, haemorrhage are thought to be due to the initiation of an inflammatory response. Various prostaglandins and cytokines are thought to be produced by the urothelial cells leading to inflammation (Gomes et al., 1995, Ribeiro et al., 2002, Hu et al., 2003, Klinger et al., 2007, Korkmaz et al., 2007, Macedo et al., 2008a, Macedo et al., 2008b, Conklin et al., 2009). One study, in rats, has found that CPO induces a significant increase in bladder mRNA expression of the cytokines interleukin (IL)-1 β ,

IL-2, IL-4, IL-6 and tumour necrosis factor (TNF)- α and TNF- β mRNA after acute cystitis (4 hours after CPO treatment) (Malley and Vizzard, 2002). The protein expression of the cytokines was generally equal to that of mRNA (Malley and Vizzard, 2002). In particular, the inhibition of TNF- α and IL-1 β in mice treated with CPO induced a significant reduction in urothelial erosion, haemorrhage, oedema, leukocyte migration, fibrin deposition and ulcerations suggesting these cytokines are crucial mediators involved in the inflammatory response to CPO. Furthermore, antiserum treatments to TNF- α or IL-1 β also inhibited the expression of iNOS in the urothelium of mice in response to IFO treatment (Gomes et al., 1995, Ribeiro et al., 2002).

In addition, several studies in rats and mice have found that systemic treatment with CPO, IFO or intravesical treatment with acrolein induces COX-2 expression in urothelium and ICs within 12-24 hours of treatment (Macedo et al., 2008b, Macedo et al., 2008a, Wang et al., 2008b, Macedo et al., 2011). One study found that the plasma PGE₂ concentration was increased after IFO administration in rats and that co-administration of a COX-2 inhibitor had a protective effect against IFO induced cystitis (Macedo et al., 2011). Therefore, it appears that COX-2 and PGE₂ may also contribute to the CPO or IFO induced bladder inflammation.

Functional effects

Functional effects such as bladder pain, urinary frequency, urgency, dysuria and feelings of incomplete emptying may be due to direct damage or alteration of the bladder or nerves by reactive drug metabolites or as a result of urothelial damage or the inflammatory response. Animals treated with CPO, IFO or intravesical acrolein demonstrate typical urotoxic storage symptoms including reduced bladder capacity, increased urinary frequency and decreased contractile force leading to decreased voiding pressure (Okinami et al., 2014, Macedo et al., 2008a, Wang et al., 2008b, Kageyama et al., 2008).

Isolated bladder tissue from animals treated with CPO or IFO demonstrated reduced contraction in response to potassium chloride (KCl) suggestive of damage to bladder smooth muscle (Macedo et al., 2011, Giglio et al., 2005). However, the bladder muscle also showed reduced contractility to the muscarinic agonist carbachol as well as altered muscarinic receptor expression, indicating that functional changes may involve more than just reduced muscle contractility (Giglio et al., 2005). Similarly, contractile responses to the P2X receptor agonist β , γ -methylene ATP (β , γ -mATP) were reduced in CPO treated animals and may be due to changes in receptor expression (Mok et al., 2000, Kageyama et al., 2008).

Alterations to afferent nerve function may also lead to the bladder pain and dysfunction seen after CPO or IFO treatment. Studies using isolated DRG from animals treated with CPO have

demonstrated increased firing rate to stimulus and activation at lower stimulus thresholds suggesting that nerves may become sensitised and hyperactive after CPO treatment (Yoshimura and de Groat, 1999, Dang et al., 2008).

Uroprotection

Mesna (sodium-2-mercaptoethanesulfonate) is a thiol compound that is commonly used in combination with CPO or IFO to reduce or ameliorate the urotoxic symptoms of these drugs. Mesna, once activated to dimesna, has free sulfhydryl groups that can combine directly with the double bond of acrolein thereby inactivating acrolein and preventing it from damaging the bladder (Stofer-Vogel et al., 1993). Shepherd and colleagues (1991) treated 100 patients undergoing bone marrow transplant conditioning with regimens that included high-dose cyclophosphamide randomly with either mesna or forced saline diuresis to study how effective mesna is in preventing hemorrhagic cystitis. The incidence of consistent or severe haematuria was 33% in the mesna treated patients as opposed to 20% in the hyperhydrated patients ($P = 0.31$) (Shepherd et al., 1991). Furthermore, Lima et al., (2007) studied the histological changes in bladders of patients treated with ifosfamide in combination with mesna and found that even after use of mesna, 66.7% of patients presented cystoscopic alterations and 100% showed microscopic alterations in the urothelial/LP such as oedema, exocytosis, and haemorrhage.

Sakurai and colleagues, (1986) treated patients receiving high-dose ifosfamide with or without mesna to investigate the effect of mesna on urotoxic side effects. Although mesna appeared to reduce the incidence of haematuria, other symptoms, such as frequency and dysuria were not statistically different between the groups. This suggests that despite the clinical use of mesna, a significant percentage of patients treated with CPO or IFO are still likely to suffer from urotoxic side effects of these drugs. Accordingly, additional or alternative protective agents are required.

Summary

In summary, we are only starting to understand the changes induced by CPO, IFO, acrolein and CAA in the bladder. It is known that acrolein has adverse effects on the bladder, causing bladder inflammation and hyperactivity resulting in micturition pain, frequency and urgency. However, despite CAA being linked to the neuro-, cardio- and nephrotoxicity of these drugs, it has received little attention in relation to urotoxicity. Additionally, despite the clinical use of mesna, a significant percentage of patients treated with CPO or IFO are likely to suffer from urotoxic side effects of these drugs. Therefore, studies into the mechanisms of cytotoxic action of CPO and IFO and the functional changes they cause in the bladder are needed. This may uncover potential targets for preventing, alleviating or treating the adverse urological effects of these drugs potentially leading to better drug toleration and better treatment outcomes.

1.5 AIMS

The overall aim of this thesis was to increase our understanding of the bladder toxicity caused by the commonly used chemotherapeutics cyclophosphamide and ifosfamide and the implications of this toxicity on bladder function. Understanding how these drugs cause bladder damage and what functional changes occur as a result may reveal future targets for preventing the adverse effects of these drugs or restoring normal bladder function after treatment. This thesis used various experimental approaches to investigate the following aims:

1. To determine whether acrolein or chloroacetaldehyde are toxic to urothelial cells and whether urothelial function changes as a result.
2. To investigate whether acrolein or chloroacetaldehyde induced urothelial toxicity can be prevented by vitamin C or N-acetylcysteine.
3. To explore whether acrolein or chloroacetaldehyde can cause functional changes in the detrusor.
4. To investigate the effect of systemic cyclophosphamide and ifosfamide treatment on bladder afferent nerve activity.
5. To examine the possible interaction between changes in urothelial function and detrusor or afferent nerve function.

CHAPTER 2: Materials and Methods

This Chapter provides general information on the materials and methods used throughout the following studies. Specific and detailed information regarding the experimental protocol used in each study is detailed in the relevant Chapters.

2.1 SOLUTIONS

The composition of Krebs-bicarbonate solution was (in mM): NaCl 118, NaHCO₃ 24.9, CaCl₂ 1.9, MgSO₄ 1.15, KCl 4.7, KH₂PO₄ 1.15, and D-glucose 11.7. Hypotonic Krebs solution contained half the NaCl (59mM) while all other components were at the same concentration as for normal Krebs solution. All reagents were obtained from Sigma Aldrich Co. LLC (St. Louis, USA). The carbogen gas (95% O₂ and 5% CO₂) was obtained from BOC Gases Australia Ltd. (Australia).

The cell culture medium, McCoys 5A, was obtained in powder form containing L-glutamine and phenol red (Sigma, St. Louis, USA). The powder was reconstituted in distilled water and supplemented with 2.2 g/L sodium bicarbonate (Sigma), 10% fetal bovine serum (Life Technologies, of USA origin) and 500 U/mL penicillin-streptomycin (Invitrogen™). Other solutions used during cell culture included 0.25% trypsin and phosphate buffered saline from Life Technologies (Auckland, New Zealand).

2.2 DRUGS

All of the drugs used in the following studies, including their source and solvent, are listed in **Table 2.1**. The concentration of drugs used was either determined using preliminary experiments or derived from the concentrations used in the literature. All drugs were obtained from Sigma-Aldrich (St. Louis, USA), except those indicated (*), which were obtained from Tocris Bioscience (Bristol, UK) and those indicated (**) which were from Cayman Chemical Company (Michigan, USA).

Table 2.1: Details of the pharmacological agents used in this thesis			
Compound	Main Action	Solvent	Cat No.
1,1-Dimethyl-4-phenylpiperazinium iodide (DMPP)	Non-specific nicotinic agonist	H ₂ O	D5891
1-[3',4'-Dihydroxyphenyl]-2-isopropyl-aminoethanol (isoprenaline)	β-adrenoceptor agonist	H ₂ O	I5627
2',7'-Dichlorofluorescein diacetate (DCF-DA)	Cell permeable probe for ROS	DMSO	D6883
3-Methyladenine (3MA) **	Autophagy inhibitor	Culture Medium	13242
α,β-Methyleneadenosine 5'-triphosphate lithium salt (αβm-ATP)	P2X receptor agonist	H ₂ O	M6517
Acetylcholine chloride	Cholinergic receptor agonist	H ₂ O	A6625
Acrolein	Reactive metabolite of cyclophosphamide and ifosfamide	H ₂ O	01679
Adenosine 5'-triphosphate disodium salt (ATP)	P2 purinoceptor agonist	H ₂ O	A7699
ARL 67156 trisodium salt	ATP'ase inhibitor	H ₂ O	A265
Atropine Sulfate Salt	Non-selective muscarinic antagonist	H ₂ O	A0257
Capsaicin	TRPV1 agonist	Ethanol	M2028
Carbamylcholine chloride (carbachol)	Cholinergic receptor agonist (muscarinic > nicotinic)	H ₂ O	C240-9
Chloroacetaldehyde solution (50 wt. % in H₂O)	Reactive metabolite of cyclophosphamide and ifosfamide	H ₂ O	317276
Cyclophosphamide monohydrate	Commonly used chemotherapeutic agent	H ₂ O	C0768
Ifosfamide	Commonly used chemotherapeutic agent	H ₂ O	I4909
L-Ascorbic acid (Vitamin C)	Antioxidant	H ₂ O	A4403
N-Acetyl-L-cysteine (NAC)	Antioxidant and GSH precursor	H ₂ O	A9165
Nifedipine	L-type Ca ²⁺ channel blocker	Ethanol	N7634
N^ω-Nitro-L-Arginine (L-NNA)	Nitric oxide synthase inhibitor	H ₂ O	N-1522
Polygodial *	TRPA1 agonist	Ethanol	3197
Prostaglandin E₂	E prostanoid receptor agonist	Ethanol	P0409
Resazurin sodium salt	Probe for detection of metabolically active cells	H ₂ O	R7017
Sodium Nitroferricyanide (Sodium Nitroprusside [SNP])	Nitric oxide donor	H ₂ O	S0501
Tetrodotoxin	Na ⁺ channel blocker	Citrate Buffer (ph 4-5)	T8024
WS-12 *	TRPM8 agonist	Ethanol	3040

2.3 ASSAYS

The following assays were used in each of the studies in this thesis. Other assays used specifically in one study only are described in the appropriate Chapter.

ACh

Concentrations of ACh in the various samples collected were measured using an Amplex Red ACh/AChEsterase Assay Kit (Molecular Probes, Cat # A12217). In the assay, ACh esterase (AChE) converts ACh to choline which is in turn oxidised by choline oxidase to betaine and hydrogen peroxide (H_2O_2). In the presence of horseradish peroxidase, H_2O_2 reacts with the amplex red reagent to produce the highly fluorescent product resorufin. Equal volumes of sample (50 μ l) and reaction solution (50 μ l) (containing AChE, choline oxidase, horseradish peroxidase and amplex red in a buffer) were incubated at room temperature protected from light for 30 minutes according to the recommended protocol. Fluorescence by resorufin was then measured using a Modulus Microplate Multimode Reader (Turner Biosystems, California, USA) (excitation 571nm and emission 585nm). As the assay measures the metabolite choline, the assay calculates both the current and metabolised ACh in the sample. ACh content in samples was calculated from a standard curve of fluorescence plotted using known concentrations of ACh (**Figure 2.1**).

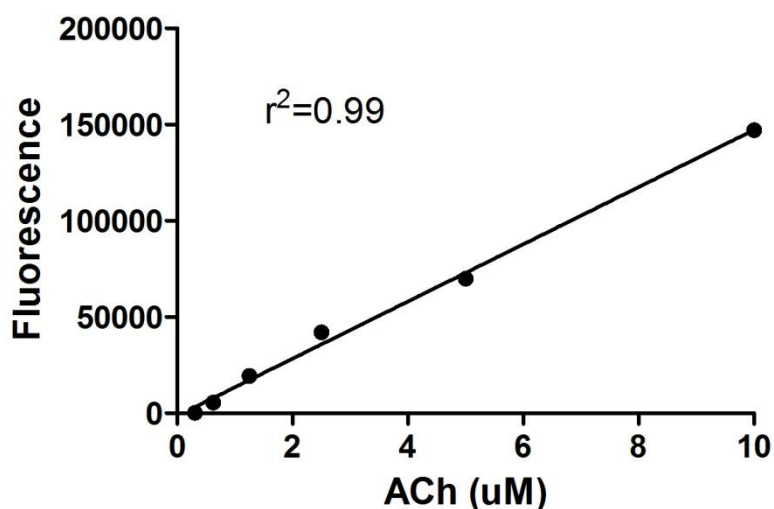
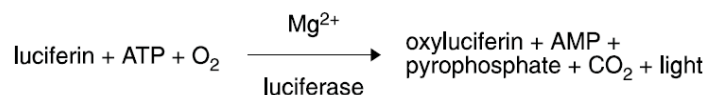


Figure 2.1: Representative standard curve of fluorescence readings to known concentrations of ACh.

ATP

Concentrations of ATP in the various samples collected were measured using a luminescence based ATP Determination Kit (Molecular Probes, Cat #A22066). The assay is based on a reaction of the enzyme firefly luciferase converting the substrate D-luciferin, in an ATP dependant reaction, to a product and light (emission maximum is approximately 560nm at pH 7.8). Luciferase requires ATP to produce light and thereby the luminescence of the reaction directly reflects the amount of ATP present in the sample.



The kit requires that reaction solution (containing firefly luciferase, D-luciferin, and dithiothreitol in a buffer) be combined with sample so that the volume of sample amounts to no more than 10% of the total assay volume. Accordingly, 10µl of sample was combined with 100µl of reaction solution and incubated at room temperature for 5 minutes before reading the luminescence. Luminescence was measured using a Modulus Microplate Multimode Reader (Turner Biosystems, California, USA) and ATP content in samples was calculated from a standard curve of luminescence plotted using known concentrations of ATP (**Figure 2.2**).

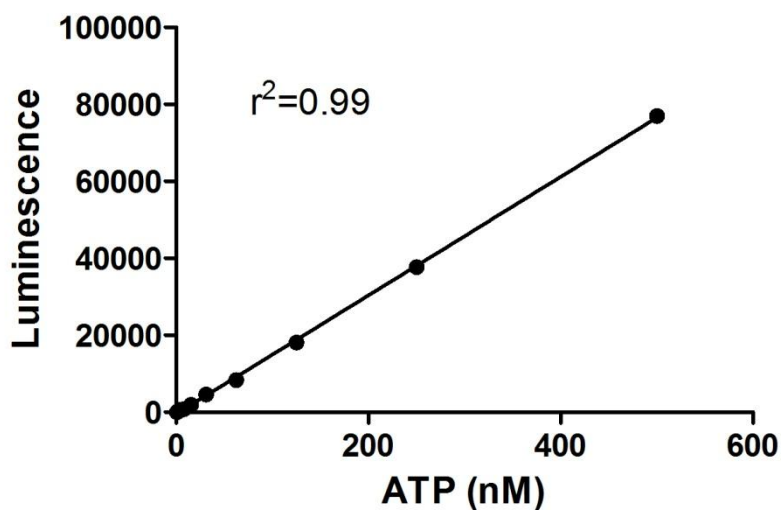


Figure 2.2: Representative standard curve of luminescence readings to known concentrations of ATP.

PGE₂

The kit used to determine PGE₂ levels in samples was the Cayman's PGE₂ EIA Kit - Monoclonal (Cat #514010). This assay uses a 96 well plate coated with goat polyclonal anti-mouse IgG as well as PGE₂ monoclonal antibodies, PGE₂-acetylcholinesterase conjugate (PGE₂ Tracer) and AChE substrate which produces a coloured product measureable by spectrophotometry. The PGE₂ Tracer (50µl) was added to each well along with sample (50µl) and PGE₂ monoclonal antibody (50µl). The PGE₂ Tracer and the PGE₂ in the sample compete for the limited amount of monoclonal antibody. The antibody-PGE₂ complex binds to the goat polyclonal anti-mouse IgG attached to the well. After incubation for 18 hours at 4°C, the plate was washed five times with a buffer to remove any unbound reagents. Next, Ellmans reagent (200µl) (which contains the substrate to AChE) was added to the well and incubated at room temperature for 30 minutes on an orbital shaker (300rpm) protected from light. The product of this enzymatic reaction has a distinct yellow colour and absorbance was measured at 412 nm using a Modulus Microplate Multimode Reader (Turner Biosystems, California, USA). The intensity of the colour is proportional to the amount of PGE₂ Tracer bound to the well, which is inversely proportional to the amount of sample PGE₂ present in the well during the incubation. PGE₂ content in samples was calculated from a standard curve of absorbance plotted using known concentrations of PGE₂ (Figure 2.3).

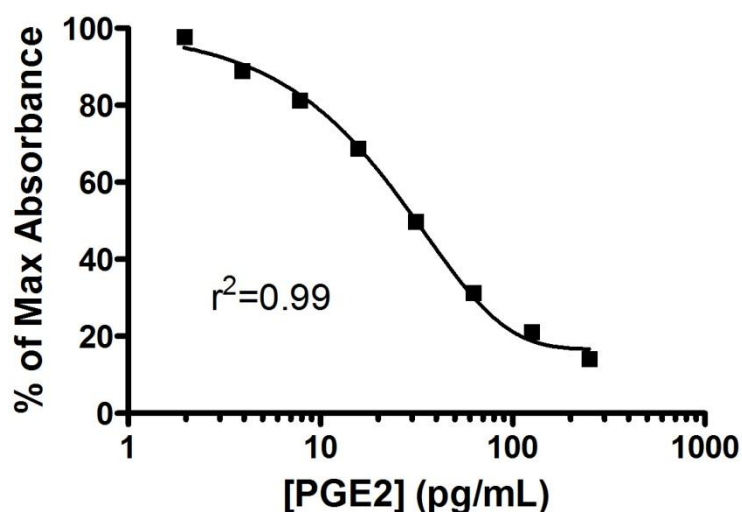


Figure 2.3: Representative standard curve of % of maximum absorbance readings to known concentrations of PGE₂.

2.4 HISTOLOGY

Histological chemistry was performed on various tissues including porcine and murine bladder tissues. Porcine tissues were dissected to an approximate size of 5mm long, 3mm wide and 3mm thick. Murine bladders were approximately 1-2mm thick. All histology processes were performed according to the following protocol.

1. Fixing: Tissues were placed into neutral buffered formalin (10%) and left for 24 hours at 4°C. For whole mouse bladder a catheter was secured in the urethra and formalin was injected into the bladder to gently distend the bladder and then placed in formalin to fix the tissue in a distended state.
2. Processing: The tissues were removed from the formalin and placed in the following solutions:
 - a. 70% ethanol (15 minutes for murine tissue, 30 minutes for porcine tissue),
 - b. 90% ethanol (15 minutes for murine tissue, 30 minutes for porcine tissue),
 - c. 100% ethanol (15 minutes for murine tissue, 30 minutes for porcine tissue),
 - d. 1:1 xylene:ethanol (7 minutes for murine tissue, 15 minutes for porcine tissue),
 - e. 100% xylene (7 minutes for murine tissue, 15 minutes for porcine tissue),
 - f. Fresh 100% xylene (7 minutes for murine tissue, 15 minutes for porcine tissue),
 - g. 1:1 xylene:paraplast at 57°C (15 minutes for murine tissue, 30 minutes for porcine tissue),
 - h. Liquid paraplast at 57°C (15 minutes for murine tissue, 30 minutes for porcine tissue), and
 - i. Change paraplast at 57°C (15 minutes for murine tissue, 30 minutes for porcine tissue).

Tissues were positioned in aluminium moulds and completely covered with liquid paraplast. A plastic cassette was placed in the paraplast and left for 12 hours to set.

3. Mounting: Tissue sections were cut at 4-5µm using an Accu-Cut[®] SRM[™] 200 Rotary Microtome (Sakura Finetek Europe B.V., The Netherlands). Sections were placed in a small water bath at 48°C and then gently positioned on a glass microscope slide and left until dry.

4. Rehydrating: Slides were placed in a heat box for 5-10 minutes and then placed in the following solutions:
 - a. 100% xylene for 1 minute,
 - b. Fresh 100% xylene for 1 minute,
 - c. 1:1 ethanol:xylene for 3 minutes,
 - d. 100% ethanol for 3 minutes,
 - e. 90% ethanol for 3 minutes,
 - f. 70% ethanol for 3 minutes,
 - g. 50% ethanol for 3 minutes, and
 - h. Distilled water for 3 minutes.
5. Staining: Slides were placed in the following solutions:
 - a. Haematoxylin stain (mayers) for 4 minutes
 - b. Distilled water for 30 seconds
 - c. Acid alcohol (0.5 or 1%) for 30 seconds
 - d. Distilled water for 30 seconds
 - e. Scott's Blue for 30 seconds
 - f. Distilled water for 30 seconds
 - g. Eosin stain for 2 minutes
 - h. Distilled water for 30 seconds
6. Dehydrating: Stained slides were then placed in the following solutions:
 - a. 50% ethanol for 3 minutes
 - b. 70% ethanol for 3 minutes
 - c. 90% ethanol for 3 minutes
 - d. 100 % ethanol for 3 minutes
 - e. Fresh 100% xylene for 1 minute
7. Visualisation: Slides were allowed to dry and then visualised under a microscope (Infinity 2, Olympus, Tokyo). Photographs were taken using an Infinity 2 microscope camera (Olympus, Japan) attached to a computer using Infinity Capture software (version 5.0.2 Lumenera Corporation, Canada).

All histology solutions and chemicals were Fronine Pathology products obtained from Thermo Fisher Scientific, Taren Point, Australia.

2.5 STATISTICAL ANALYSIS

Various statistical tests were used where appropriate and details of analysis are stated in the relevant sections of each Chapter. Statistical differences were considered significant at $p < 0.05$. All graphical analyses used in this thesis were performed using GraphPad Prism (Version 5.04 for Windows, Graph Pad Software, San Diego, USA) and all statistical analyses were performed using GraphPad InStat (version 3.06 for Windows, GraphPad Software, San Diego, USA). Normality of distribution was determined using InStat during statistical analyses and parametric or non-parametric methods used according to whether results were normally distributed or not. In the cases where the n-value was too small for InStat to accurately measure distribution of the data, the more powerful program IBM® SPSS® Statistics (version 19, SPSS Inc., an IBM Company) was used to test the normality of distribution. The test used in each analysis is stated in figure legends.

CHAPTER 3: Effects of Cyclophosphamide, Ifosfamide, Acrolein or Chloroacetaldehyde on Cultured Human Urothelial Cells

3.1 INTRODUCTION

Urothelial Function

As discussed in Chapter 1, the urothelium is important for normal function of the bladder. Firstly, it provides a barrier protecting the bladder from toxins, pH and bacteria present in the urine. It also has a complex role in sensing and releasing various mediators which may regulate detrusor contraction (Hawthorn et al., 2000) and mechanosensation (Birder, 2010). The urothelium responds to bladder filling by releasing chemical mediators such as ATP, acetylcholine (ACh), prostaglandin E₂ (PGE₂) and cytokines to communicate with underlying cells such as sensory nerves, interstitial cells and smooth muscle (Birder et al., 2010b). Disruption of these mechanisms are associated with bladder dysfunction (Kumar et al., 2010, Chen et al., 2003, Sun and Chai, 2006, Sun et al., 2001).

CPO & IFO

CPO and IFO are chemotherapeutics commonly used to treat various cancers and autoimmune conditions and cause urotoxic side effects such as bladder haemorrhage, pain, urgency and frequency. CPO and IFO are pro-drugs that undergo activation in the liver producing their active forms as well as various metabolites including acrolein and chloroacetaldehyde (Furlanut and Franceschi, 2003, de Jonge et al., 2005). Acrolein is excreted in the urine and as a result comes into direct contact with the urothelium potentially altering bladder function or compromising the urothelial barrier allowing toxins to pass into the bladder wall (Al-Rawithi et al., 1998, Brock et al., 1979). However chloroacetaldehyde (CAA) is also excreted in the urine (Shaw et al., 1983) and although it has been linked to the neuro-, cardio- and nephro-toxicity of these drugs (Goren et al., 1986, Sladek, 1988, Joqueviel et al., 1997), its possible contribution to uro-toxicity has been ignored.

It has been suggested that the main mechanism by which acrolein causes bladder damage is through the production of reactive oxygen species (ROS) and nitric oxide (NO), depleting intracellular glutathione (GSH) and then causing oxidative damage (Korkmaz et al., 2007) and leading to cell death by apoptosis or necrosis (Beauchamp et al., 1985, Kehrer and Biswal, 2000, Korkmaz et al., 2007). CAA toxicity has been suggested to occur by similar mechanisms including depletion of cellular GSH, NAD and ATP, disturbed Ca²⁺ signalling, and lipid peroxidation, potentially leading to cell necrosis or apoptosis and death (Sood and O'Brien, 1993, Schwerdt et al., 2006, Nissim et al., 2006). Furthermore, these metabolites would be present in urine together and their combined effects are yet to be investigated in the bladder.

Cell Death Mechanisms

Programmed cell death can occur by apoptosis, autophagy or necrosis (Galluzzi et al., 2012).

Apoptosis is an important homeostatic process that allows removal of unwanted cells during development, homeostasis and disease (Favaloro et al., 2012). Apoptotic cell death is characterised by nuclear and cytoplasmic condensation, cell membrane blebbing resulting in the release of small membrane-enclosed particles that are rapidly phagocytosed. There are two pathways that can lead to apoptosis in a cell including extrinsic and intrinsic apoptosis. Extrinsic apoptosis is initiated by extracellular molecules binding to trans-membrane death receptors (Favaloro et al., 2012). Upon ligand binding, death receptors undergo conformational changes leading to the assembly of a protein complex (Death Initiation Signalling Complex) that leads to activation of the caspase cascade. The intrinsic apoptotic pathway is activated in response to intracellular stress such as DNA damage and oxidative stress (Favaloro et al., 2012). These stressors converge on the mitochondria and influence mitochondrial membrane potential leading to cessation of ATP production and release of various proteins that result in activation of the caspase cascade. Accordingly, caspases are essential in the execution of apoptosis and in particular caspase-3 is an early indicator of cell death by apoptosis (Porter and Janicke, 1999).

Necrosis is different from apoptosis in that it involves mitochondrial swelling and plasma membrane rupture. Necrosis was considered an accidental cell death mechanism caused by cell trauma however, recent work has shown that necrosis can occur in a regulated manner (Galluzzi et al., 2012). Cell stress, such as DNA damage, excitotoxins and binding of death receptors can trigger necrosis. Necrosis does not always exhibit the same features but generally starts with clumping of chromatin and swelling of organelles, followed by swelling of the cell, and the rupture of nuclei, mitochondria, and the plasma membrane (Searle et al., 1982). Cells also tend to develop increased cytosolic Ca^{2+} , increased ROS and a depletion of ATP during necrosis (Golstein and Kroemer, 2007).

Autophagy is a process of intracellular degradation that occurs at basal levels in virtually all cells, performing repair and stress response functions (Glick et al., 2010). It balances energy sources in the cell during times of stress and can remove misfolded proteins, damaged cell organelles and pathogens. Organelles and proteins are sequestered into autophagic vesicles that then fuse with lysosomes and are degraded thereby preventing accumulation of damaged components or recycling unnecessary parts to maintain energy homeostasis. Autophagy is generally thought of as a survival mechanism given its housekeeping role in the cell as well as

its ability to protect against genome instability and necrosis, however deregulation of autophagy has been linked to non-apoptotic cell death (Glick et al., 2010). The activity of caspase-3 and autophagy were measured in this study to investigate the effects of acrolein and CAA on cell death pathways.

Inflammation in the bladder

Painful bladder syndrome (PBS) (also interstitial cystitis or bladder pain syndrome) is a disease with very similar urinary symptoms to CPO/IFO urotoxicity such as increased urinary frequency, urgency and suprapubic pain (Parsons, 2007). The cause of PBS is still unknown however evidence suggests that afferent sensitisation as a result of bladder inflammation, production of inflammatory cytokines/chemokines and urothelial dysfunction is involved in the development of symptoms (Amaravadi et al., 2011, Erickson et al., 2002, Keay, 2008). Inflammation has been shown in biopsies in many patients diagnosed with PBS (Erickson et al., 2008, Wyndaele et al., 2009). Furthermore, PBS patients have increased cytokine and chemokines in their urine including IL-2, IL-6, IL-8 and TNF- α (Erickson et al., 2002, Keay, 2008, Ogawa et al., 2010, Peters et al., 1999).

Inflammation is associated with the release of irritant chemicals or inflammatory mediators from the urothelium or afferent nerves, such as ATP (Burnstock, 2013, Smith et al., 2005), PGE₂ (Maggi, 1990, Maggi, 1992) and neurotrophic factors such as NGF (Dmitrieva and McMahon, 1996, Ochodnický et al., 2011), that can activate or sensitise afferent nerves. Bladder inflammation has been reported in response to intravesical instillation of various toxic substances including hydrogen peroxide and protamine sulphate which induce bladder overactivity and dysfunction (Homan et al., 2013, Lv et al., 2013). Accordingly, it is possible that CPO and IFO induced cystitis involves a similar inflammatory response and the question remains as to whether acrolein and/or CAA contributes to this mechanism.

Effects of CPO & IFO on the Urothelium

Within 24 hours of treatment with CPO, the urothelium suffers widespread destruction by necrosis and apoptosis of the urothelial cells (Lee et al., 2014). It is thought that acrolein is to blame for the damaging effects of CPO and IFO. In support of this, acrolein (2 μ M) has been shown to reduce proliferation of cultured rat urothelial cells by almost 60% after 24 hours (Nirmal et al., 2014). However, to my knowledge, these effects have not been confirmed in human urothelial cells. Furthermore, no studies have looked at whether CAA causes urothelial cell damage.

One study suggests that reduction of uroplakin expression is a contributing factor to the damage caused by CPO and IFO (Kyung et al., 2011). In bladders from rats treated with CPO, the level of all uroplakin mRNA and the protein expression of UP II and IIIa were maximally suppressed within 12 hours and partial and complete recoveries were seen at 24 and 72 hours post administration respectively.

Changes in mediator release from the urothelium have been reported in various painful or overactive bladder conditions. Specifically, stretch-induced ATP release has been reported in tissue samples from patients with PBS (Chen et al., 2003, Sun and Chai, 2006, Sun et al., 2001) and bladder overactivity (Kumar et al., 2010). Rats treated with CPO have also demonstrated increased urothelial ATP release in response to hypo-osmotic stimulation (Smith et al., 2005). Any changes in ACh release are yet to be assessed.

Furthermore, several studies in rats and mice have found that systemic treatment with CPO, IFO or intravesicular treatment with acrolein induces COX-2 expression in urothelium within 12-24 hours of treatment (Macedo et al., 2008b, Macedo et al., 2008a, Wang et al., 2008b, Macedo et al., 2011). One study found that the plasma PGE₂ concentration was increased after administration of IFO in rats and that co-administration of a COX-2 inhibitor had a protective effect against IFO induced cystitis (Macedo et al., 2011). Therefore, it appears that COX-2 and PGE₂ may also contribute to the acrolein induced bladder inflammation.

Protection of the Urothelium

As discussed previously, mesna is commonly used in combination with CPO or IFO to inactivate acrolein and prevent it from damaging the bladder (Stofer-Vogel et al., 1993). However, several studies have found that despite the clinical use of mesna, a significant percentage of patients treated with CPO or IFO are still likely to suffer from uro-toxic side effects of these drugs (Lima et al., 2007, Shepherd et al., 1991, Sakurai et al., 1986). Accordingly, alternative or additional methods of protecting the bladder from CPO and IFO toxicity are required.

The mechanism of toxicity of acrolein and CAA is via the production of ROS and NO leading to GSH depletion and oxidative damage. In addition, both molecules have the ability to react with proteins, lipids and potentially DNA causing direct damage themselves. Accordingly, the use of antioxidants or GSH precursors has been suggested as a logical method of protecting the bladder from oxidative damage after GSH depletion. Numerous different agents have been tested for protective effects against CPO induced cystitis. Some promising results were demonstrated using flavanoid antioxidants either alone or in combination with mesna (Ozcan et

al., 2005, Sadir et al., 2007). The antioxidant and GSH precursor N-acetylcysteine (NAC) has also shown promising results protecting mice urothelial cells from genotoxic damage due to CPO treatment (Gurbuz et al., 2009). In addition, NAC has also been shown to protect Chinese hamster ovary cells from apoptosis due to acrolein (Tanel and Averill-Bates, 2007). Therefore, NAC may protect urothelial cells from functional changes due to CPO and IFO toxicity and should be further investigated in human tissues. A common nutritional antioxidant, vitamin C, has received little attention in relation to preventing CPO or IFO induced cystitis. Given the success of antioxidants in protecting the bladder and the relative safety and availability of vitamin C, it also warrants further investigation.

Evaluation of Urothelial Function

Urothelial cell culture provides an invaluable *in vitro* model for investigating biological processes at the cellular level. Two commonly used human urothelial cell lines include the RT4 and T24 cells. The RT4 cell line is derived from a non-malignant transitional cell papilloma of human urinary bladder, demonstrating growth and motility characteristics of normal epithelial cells (Rigby and Franks, 1970, Kabaso et al., 2011). Whereas, the T24 cell line is derived from a transitional cell carcinoma of human urinary bladder, showing malignant behaviour including spreading and separating during cell motility (Bubenik et al., 1973, Kabaso et al., 2011). RT4 cells have similar size and shape and form tight connections between cells while T24 cells grow less uniformly with varied size and shape and tend to grow one on top of another (Kabaso et al., 2011). The RT4 cell line expressed all four uroplakin transcripts, whereas less differentiated cell lines lose their uroplakin expression (Lobban et al., 1998) with expression in T24s being undetectable (Huang et al., 2007).

RT4 cells have been shown to release ATP during basal (unstimulated) conditions with release increasing during hypotonic stimulation (Mansfield and Hughes, 2014, Kang et al., 2013, McDermott et al., 2012). The RT4 cells have also been shown to release ACh and PGE₂ in basal and hypotonic conditions (McDermott et al., 2012, Kang et al., 2013). Accordingly, the RT4 cells appear to be a good model for studying the function of normal urothelial cells. No studies have measured release of mediators from T24 cells however, they may also be useful for investigations into urothelial function and whether the loss of normal urothelial interactions affects function.

Aims

The aim of the present study was to investigate the urothelial cell viability and function after treatment with CPO, IFO, acrolein or CAA. Specific aims were:

- To determine the effect of acrolein on viability of human urothelial cells;
- To determine if CAA is toxic to human urothelial cells;
- To assess if ATP, ACh and PGE₂ release from human urothelial cells is changed by acrolein or CAA treatment;
- To investigate whether acrolein or CAA treatment affects autophagy or caspase-3 activation;
- To determine if vitamin C or N-acetylcysteine protect urothelial cells from damage by acrolein or CAA.

3.2 METHODS

Cell Lines

The established cell lines RT4 and T24 were used to study urothelial function. The cell lines were obtained from the European Collection of Cell Cultures and stored in freeze medium containing DMSO (10%) in liquid nitrogen until required. The cells were maintained in McCoy's 5A culture medium (Sigma Aldrich, St. Louis, USA) containing L-glutamine, phenol red, 10% fetal bovine serum and 500 U/ml penicillin-streptomycin (Invitrogen™), as described previously (McDermott et al., 2012). All cultures were maintained at 37°C in a humidified atmosphere of 5% CO₂ in air and passaged at approximately 90% confluence by detachment with 0.25% (w/v) trypsin for 2-4 minutes. Cells were routinely passaged every 3-4 days with a typical split of 1:4 for RT4 and 1:8 for T24 cells respectively. **Figure 3.1** shows the different growth pattern of the RT4 (A) and T24 (B) cells.

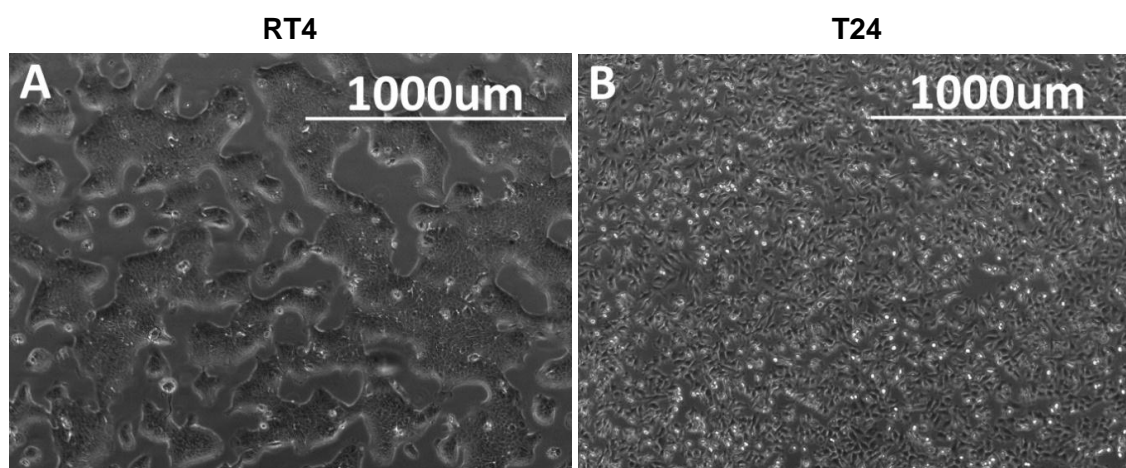


Figure 3.1: RT4 (A) and T24 (B) human urothelial cell lines at magnification of 40x.

Compound Screening Concentrations

The parent drugs CPO and IFO are not cytotoxic themselves and therefore were not expected to be directly implicated in the urotoxicity of CPO and IFO treatment (Furlanut and Franceschi, 2003, de Jonge et al., 2005). However, initial screening of the drugs was performed to ensure that this assumption was correct and that CPO and IFO are not damaging urothelial cells. The toxic metabolites of CPO and IFO, acrolein and chloroacetaldehyde, were investigated for their effects on urothelial function.

As discussed previously, very little is known about the actual concentrations of acrolein and CAA appearing in the urine of patients treated with cyclophosphamide and ifosfamide. The excretion of these drugs and their metabolites varies from patient to patient as well as between subsequent treatments within the same individual. In addition, the initial level of hydration and the ongoing fluid intake of the patient are likely to lead to variable urine volumes and urine concentrations of the drugs and metabolites. Accordingly, a range of concentrations are likely to be relevant in the urotoxicity of CPO and IFO. Therefore, this study investigated a range of concentrations of CPO, IFO, acrolein and chloroacetaldehyde extrapolated from available urine and plasma concentration data discussed in Chapter 1. The concentration range that was initially investigated for each substance was 10nM-100µM. This range is consistent with previous studies which have tested concentrations of IFO, CPO, acrolein and CAA of 15-300µM in human proximal tubule cells (Schwerdt et al., 2006), 5-100µM of acrolein in human bronchial epithelial cells (Nardini et al., 2002) and 50-500µM of CAA and acrolein in human hepatocytes (Macallister et al., 2012). Acrolein has also been instilled directly into bladders of mice at a concentration of 400µM in order to induce cystitis (Wang et al., 2008b). Again, as outlined above, in most CPO and IFO treatment regimens, very little excretion of the parent drug and its metabolites is observed after 24 hours. Therefore, drug treatments in this study used a single concentration over a 24 hour period.

Experimental Setup and Procedures

To prepare experiments with the RT4 and T24 cell lines, 24 or 96 well plates or T25 flasks were seeded with cells at a density determined during initial optimisation experiments, see **Table 3.1**. Cell number and cell viability was assessed by trypan blue exclusion. The cells were then left for 24 hours to attach before being treated with one of CPO, IFO, acrolein or chloroacetaldehyde for a further 24 hours. After treatment various tests were performed to determine changes in viability and function.

Table 3.1 – Typical seeding densities used for experiments with RT4 and T24 cell lines				
Cell Line	Seeding Density in 96 well plates	Seeding Density in 24 well plates	Seeding Density in T25 Flasks	Typical Split for Passaging in T75 Flasks
RT4	1.35 x 10 ⁴ per well	2 x 10 ⁵ per well	1.5 x 10 ⁶ per flask	1:4
T24	1.35 x 10 ⁴ per well	1 x 10 ⁵ per well	1 x 10 ⁶ per flask	1:8

Cell Viability Assay

Resazurin (alamar blue) is a redox dye that can be used to measure cell viability (Gonzalez and Tarloff, 2001). Resazurin can be reduced intracellularly by viable, metabolically active cells into resorufin and dihydroresorufin, with resorufin measurable flurometrically to indicate cell viability (excitation 530nm; emission 590nm) (O'Brien et al., 2000). The fluorescence of resorufin increases proportionally to the number of cells present in the well and there was a significant positive correlation between live cell number and resorufin fluorescence ($p < 0.01$) (**Figure 3.2**).

After treatment in 96 well plates, cells were washed twice with phosphate buffered saline and incubated at 37°C for 1 hour (T24 cells) or 2 hours (RT4 cells) with fresh medium containing 44µM resazurin. The reduction of resazurin to resorufin by viable cells was measured using a Modulus Microplate Multimode Reader (Turner Biosystems, California, USA). A cell free control was also included to account for any background fluorescence.

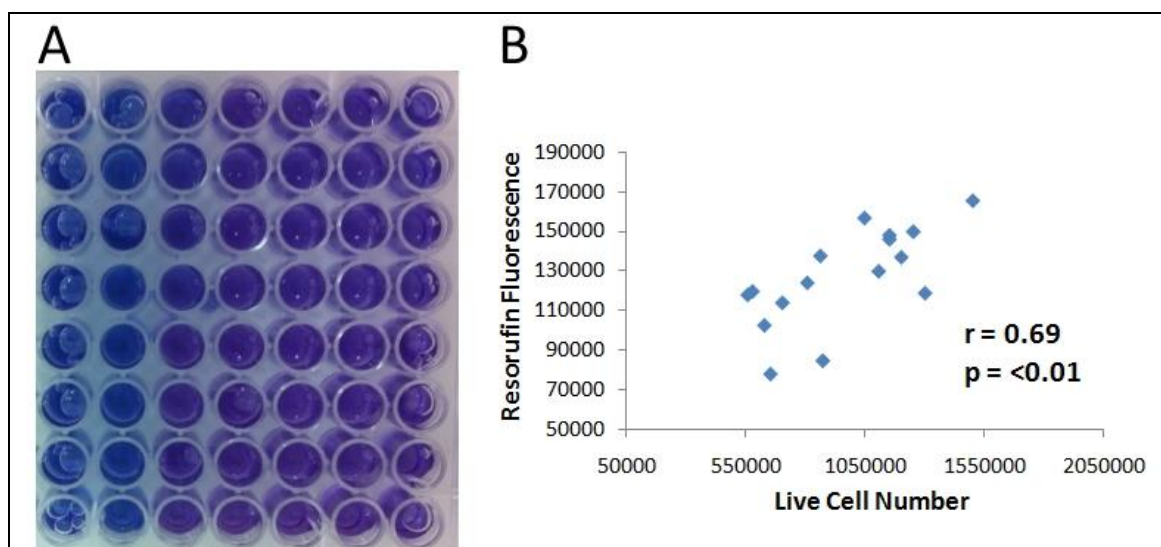


Figure 3.2: Resazurin in a 96 well plate (A) cell number increasing from left to right and Pearson correlation analysis of the relationship between live cell number and resorufin fluorescence (B).

ROS Production Assay

The probe 2',7'-Dichlorofluorescein diacetate (DCF-DA) can readily cross the cell membrane where reactive oxygen species can oxidise it, removing the acetate groups and producing a fluorescent product (DCF). This fluorescent product can then be measured using spectrometry to quantify ROS production.

Again, after treatment in 96 well plates, cells were washed with phosphate buffered saline and incubated at 37°C, protected from light for 40 mins with fresh unsupplemented medium containing 10µM DCF-DA. Fluorescence of the oxidised dye (excitation 530nm; emission at 590nm) was measured using a Modulus Microplate Multimode Reader (Turner Biosystems, California, USA) and normalised to the corresponding viability.

Caspase

Caspase-3 activity was measured using a Caspase-3 Fluorescence Assay Kit (Cayman Chemicals, Michigan, USA [Cat #10009135]). The kit uses a specific caspase-3 substrate (N-Ac-DEVD-N'-MC-R110) which produces a highly fluorescent product when cleaved by caspase-3. The fluorescence (excitation 485nm and emission 535nm) of the product was measured using a Modulus Microplate Multimode Reader (Turner Biosystems, California, USA).

RT4 and T24 cells were seeded at 2×10^4 and 1×10^4 respectively in 96 well plates and incubated for 24 hours. Cells were then treated with acrolein (100 μ M), CAA (10 μ M) or the combination of acrolein (100nM) with CAA (10 μ M) for 24 hours. The culture medium was removed and cells were washed with a buffer solution. Next, the cells in each well were lysed to release the intracellular contents (including any caspase-3 enzyme) and the cell lysates were incubated with the caspase-3 substrate at 37°C, protected from light for 30 minutes. Caspase-3 activity in cells in the absence and presence of 300 μ M NAC was also investigated. Caspase-3 activity was normalised to cell viability and represented as a percentage of control levels.

Autophagy

A Cyto-ID[®] Autophagy Detection Kit (Enzo Life Sciences, New York, USA, Cat #ENZ-51031) was used to measure autophagic activity in cultured human urothelial cells. The kit uses a novel dye that selectively labels autophagic vacuoles. The dye is a cationic amphiphilic tracer that rapidly partitions into cells and exhibits bright fluorescence upon incorporation into pre-autophagosomes, autophagosomes, and autolysosomes. Functional moieties on the dye are selective for autophagic vacuoles and prevent its accumulation within lysosomes. Nuclear material was stained using Hoechst 33342 Nuclear Stain.

RT4 and T24 cells were seeded at 2×10^4 and 1×10^4 respectively in 96 well plates and incubated for 24 hours. Cells were then treated with acrolein (100 μ M), CAA (10 μ M) or the combination of acrolein (100nM) with CAA (10 μ M) for 24 hours. The culture medium was removed from the cells and they were washed gently with phosphate buffered saline. Both the autophagic Cyto-ID stain and the nuclear Hoechst 33342 stain were added to culture medium without Phenol Red Indicator and supplemented with 5% FBS. The culture medium containing the stains was applied to each well of cells and incubated at 37°C, protected from light for 30 minutes. The culture medium was then removed and the cells gently washed twice with phosphate buffered saline before imaging. An EVOS[®] FL fluorescent microscope (Advanced Microscope Group, USA) with a standard FITC filter set was used to visualise the autophagic staining while a DAPI filter set was used to image the nuclear signal and the two images were overlaid.

Transmitter Release Assays – Optimisation

Next, transmitter release by the human urothelial cells was investigated. After treatment in 24 well plates the cells were incubated with normal Krebs bicarbonate solution and hypotonic Krebs bicarbonate solution and a sample of each solution collected and assayed for various mediators. Hypotonic Krebs can be used as an *in vitro* method for evoking mechanical stress similar to that seen with mechanical stretch or distension (Hamill and McBride, 1996, Birder et al., 2003). In this way differences in both basal release and stimulated (stretch-induced) release of the transmitters/mediators could be measured. A range of time points was initially investigated to determine the optimum point for further studies. Firstly, 150µl of normal Krebs solution (recipe on page 53) was applied to a well containing cells for 5, 10 or 15 minutes followed by the same amount of hypotonic Krebs solution (50% normal Na⁺ osmolality) for the same amount of time. As shown in **Figure 3.3** the measureable level of mediators in the samples generally increased with the incubation time. This time course had previously been performed in our lab in RT4 cells (Kang et al., 2013) but not in T24 cells. The 15 minute sample had greater levels of mediators present as well as a greater difference between basal and stimulated levels for RT4 cells and ATP in T24 cells. Accordingly, for ease of comparison the 15 minute time point was chosen for further mediator release studies. The number and viability of the cells in each well was determined both before and after the Krebs mediator collection procedure to ensure that the process was not killing or detaching cells (**Figure 3.4**). The number of cells before and after the Krebs mediator collection process did not significantly differ from each other ($p>0.05$, $n=3$ performed in quadruplicate) and the viability of the remaining cells was greater than 97%.

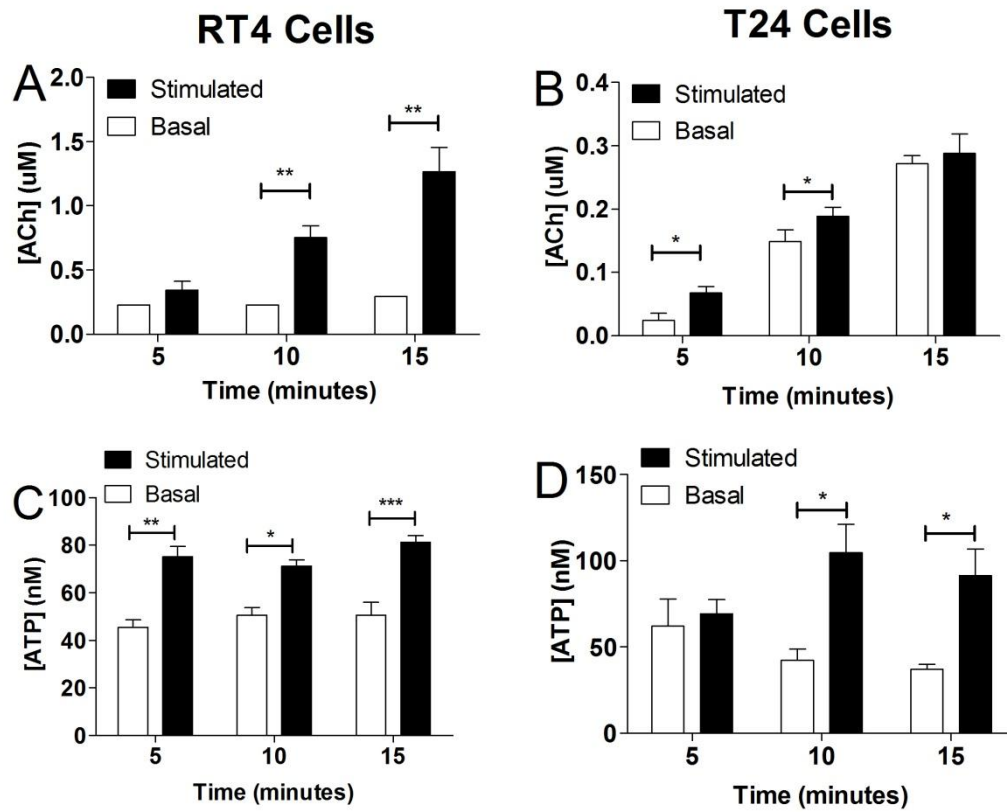


Figure 3.3: Comparison of basal and stimulated ACh and ATP release from RT4 (n=5) (A & C respectively) and T24 (n=6) (B & D respectively) cell lines (mean \pm SEM). Data analysed by unpaired two-tailed t-test * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. The RT4 time course data is from previous work (Kang et al., 2013).



Figure 3.4: Resorufin fluorescence before and after incubation with normal and hypotonic Krebs solution for 15 minutes each.

Collection of Samples for Mediator Analysis After Treatment

After treatment, each well was washed twice with Krebs solution before 150µl of normal Krebs solution was applied to the cells for 15 minutes followed by the same amount of hypotonic Krebs solution for the same amount of time. The samples of Krebs solution were collected and frozen at -20°C for later analysis using commercially available assay kits to measure the levels of ATP, ACh, and PGE₂. Samples were frozen for a maximum of three weeks before use. Detailed protocols for the assays can be found in Chapter 2. Cell viability was determined for each well after the mediator collection procedure and mediator levels were normalised to the corresponding viability. In the event that mediator concentrations were calculated from a standard curve only standard curves with an r^2 value of ≥ 0.99 were used. A lactate dehydrogenase assay was also performed to check that mediators were not simply leaking out of the cells due to increased membrane permeability after treatment.

Lactate Dehydrogenase (LDH)

The presence of LDH in samples from cultured urothelial cells was measured using a LDH Cytotoxicity Assay Kit (Cayman, Cat #10008882). The kit uses the action of LDH released into the sample solution by cultured urothelial cells to reduce NAD⁺ to NADH and H⁺ by oxidation of lactate to pyruvate. Diaphorase then uses the newly formed NADH and H⁺ to reduce tetrazolium salt producing a highly-coloured product (formazan).

RT4 and T24 cells were seeded at 2×10^5 and 1×10^5 respectively per well in a 24 well plate and left to attach overnight. Cells were then treated with acrolein (100µM) or CAA (10µM) for 24 hours. The mediator release protocol was performed as outlined above and the samples were used in the LDH kit. The normal and hypotonic krebs samples from each well were combined with a reaction solution containing NAD⁺, lactate, diaphorase and tetrazolium salt and incubated at room temperature on an orbital shaker, protected from light for 30 minutes. Absorbance of formazan was measured using a Modulus Microplate Multimode Reader at 490nm and LDH levels in treated samples were represented as a percentage of control levels.

Cytokine Release Assays

The presence of inflammatory cytokines (interleukin (IL)-8, IL-1β, IL-6, IL-10, IL-12p70 and tumour necrosis factor (TNF) in urothelial cell incubation medium was assayed using a Cytometric Bead Array Human Inflammatory Cytokine Kit (BD Biosciences) according to the manufacturer's instructions. The kit uses beads, each conjugated with a specific antibody to one of the cytokines. A detection agent comprised of a mixture of phycoerythrin-conjugated antibodies provides a fluorescent signal in proportion to the amount of bound analyte. The

beads, detection reagent and recognised cytokine form sandwich complexes (capture bead + cytokine + detection reagent) which can be measured using flow cytometry to identify particles with fluorescence characteristic of both the bead and the detector.

RT4 and T24 cells were seeded at 1.5×10^6 and 1×10^6 cells respectively in 3ml in T25 culture flasks and incubated for 24 hours. Cell free incubation medium was collected from T25 culture flasks following 24 hour treatment with either of the parent drugs or metabolites. Standard and sample fluorescence were measured on a BD FACSVerse™ flow cytometer. Concentrations of inflammatory cytokines were normalised to controls using corresponding cell numbers determined by trypan blue-exclusion.

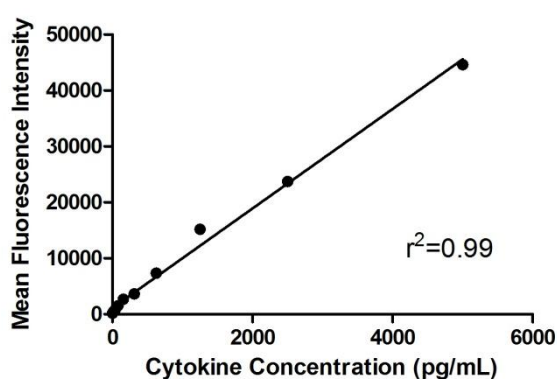


Figure 3.5: Representative standard curve of mean fluorescence intensity to known concentrations of cytokines.

Statistical Analysis

Results were expressed as mean \pm standard error of the mean (SEM). Data were analysed using a Student t-test or one-way ANOVA with Dunnett or Tukey multiple comparisons test as appropriate, using Graphpad InStat (version 3.06) software (SanDiego, CA). Significance levels were defined as $p < 0.05$ (*), $p < 0.01$ (**) and $p < 0.001$ (***).

3.3 RESULTS

Effects of CPO, IFO, Acrolein and CAA on Cell Viability and ROS

Formation

The toxicity of CPO, IFO and the metabolites acrolein and CAA was first assessed in terms of their ability to induce changes in urothelial cell viability and ROS formation. Twenty four hour exposure of either cell line to the parent compounds CPO or IFO at concentrations up to 100µM did not affect cell viability, except for 100µM CPO in T24 cells which reduced viability by 10% (Figure 3.6-A & B). Neither CPO nor IFO affected ROS formation at concentrations up to 100µM in either cell line (Figure 3.6-C & D).

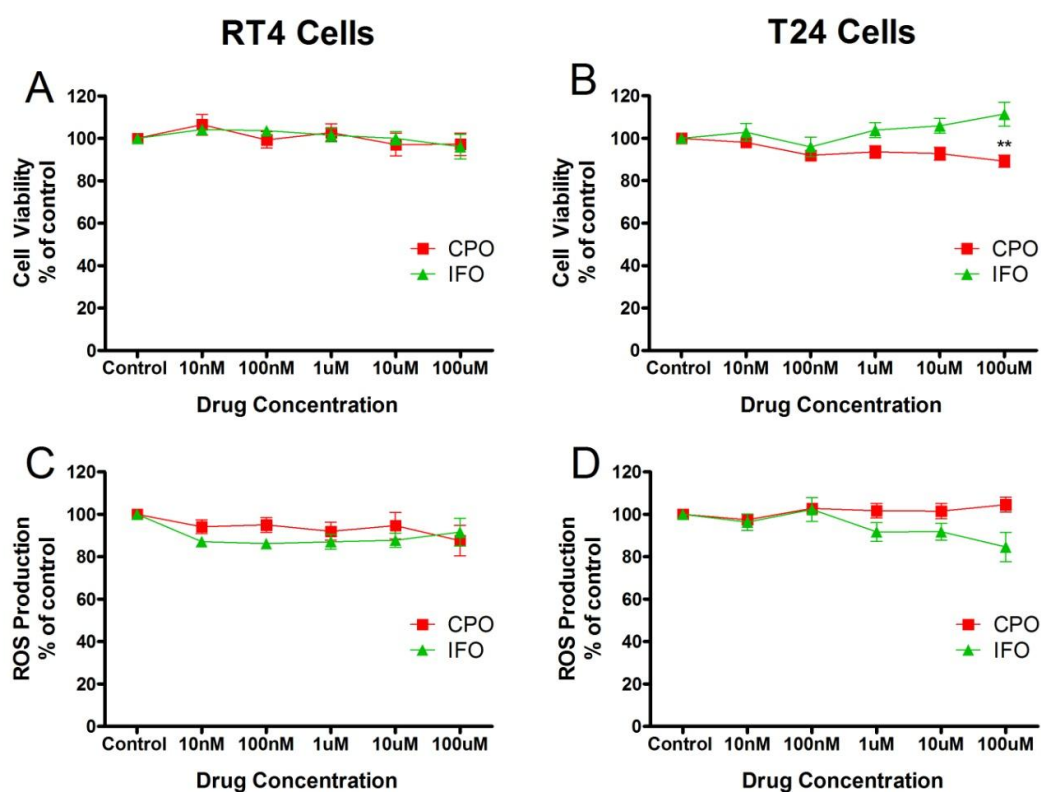


Figure 3.6: Effect of 24 hour cyclophosphamide (CPO) and ifosfamide (IFO) treatment on cell viability (A & B) and ROS production (C & D) in RT4 and T24 cells. Data are shown as a percentage of control (mean \pm SEM, n=6) and analysed by 1-way ANOVA with Dunnett post-test (**p<0.01 vs CPO control).

On RT4 cells CAA was significantly more toxic than acrolein and the mean IC_{50} value [10(8-14) μ M] was significantly lower ($p<0.0001$) than that for acrolein [67(47-95) μ M] (**Figure 3.7-A**). Similarly on T24 cells, CAA was significantly ($p<0.0001$) more toxic than acrolein [IC_{50} of CAA 4(3-5) μ M compared with 15(12-19) μ M for acrolein] (**Figure 3.7-B**).

ROS formation after acrolein or CAA treatment tended to increase as cell viability decreased. Specifically, acrolein (100 μ M) caused a 2.6-fold increase in basal ROS levels while CAA (100 μ M) resulted in a 5-fold increase in ROS production ($p<0.01$) (**Figure 3.7-C**). T24 cell viability was too low after 100 μ M acrolein or CAA treatment to successfully measure ROS levels, however at 10 μ M, acrolein and CAA caused a 30% and 3-fold increase in ROS levels respectively ($p<0.01$) (**Figure 3.7-D**). Cell viability and ROS production values can be found in **Table 3.2**.

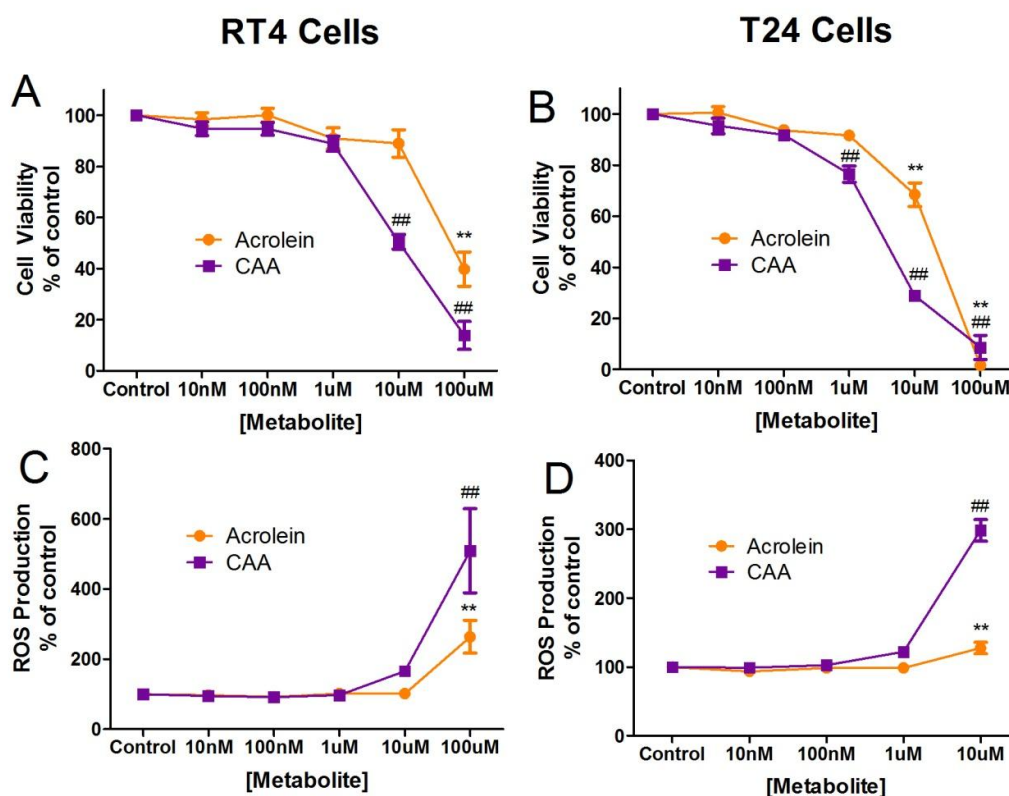


Figure 3.7: Effect of 24 hour acrolein or chloroacetaldehyde (CAA) treatment on cell viability (A & B) and ROS production (C & D) in RT4 and T24 cells. Data are shown as a percentage of control (mean \pm SEM, $n=6$) and analysed by 1-way ANOVA with Dunnett post-test (* $p<0.05$, ** $p<0.01$, *** $p<0.001$, vs acrolein control, ## $p<0.01$, ### $p<0.001$ vs CAA control).

These metabolites are present in the urine together, so possible interaction between acrolein and CAA was also investigated. To determine whether the reported urinary concentration of acrolein (100nM) (Takamoto et al., 2004) was affecting the toxicity of CAA, the toxic CAA concentration of 10 μ M was selected. The concentration of CAA (10 μ M) was chosen because it had a measurable effect on cells and therefore interaction with acrolein (100nM) could be more easily determined. In addition, this concentration is likely to appear in the urine of patients (Goren et al., 1986).

CAA (10 μ M) alone reduced cell viability in T24 cells to 37.8 \pm 1.5% of the control. The combination of acrolein (100nM) and CAA (10 μ M) caused a significant reduction in cell viability in T24 cells to 35.6 \pm 1.5% of the control which was similar to the effect seen after CAA (10 μ M) alone (**Figure 3.8-A**). However, in the RT4 cells, combined acrolein (100nM) and CAA (10 μ M) treatment reduced cell viability to 64.3 \pm 2.0% of control which was significantly greater than the viability after CAA (10 μ M) alone (50.4 \pm 2.9% of control) ($p < 0.05$).

ROS formation was also measured after treatment with the combination of acrolein (100nM) and CAA (10 μ M). Again in T24 cells the increase in ROS production after treatment with the combination of metabolites was 219 \pm 6% of control, similar to that observed with CAA (10 μ M) alone (210 \pm 7%). Whereas in RT4 cells ROS formation in the combination treated cells was 145 \pm 5% of control, significantly lower than after CAA (10 μ M) alone (167 \pm 7%) ($p < 0.01$) (**Figure 3.8-B**).

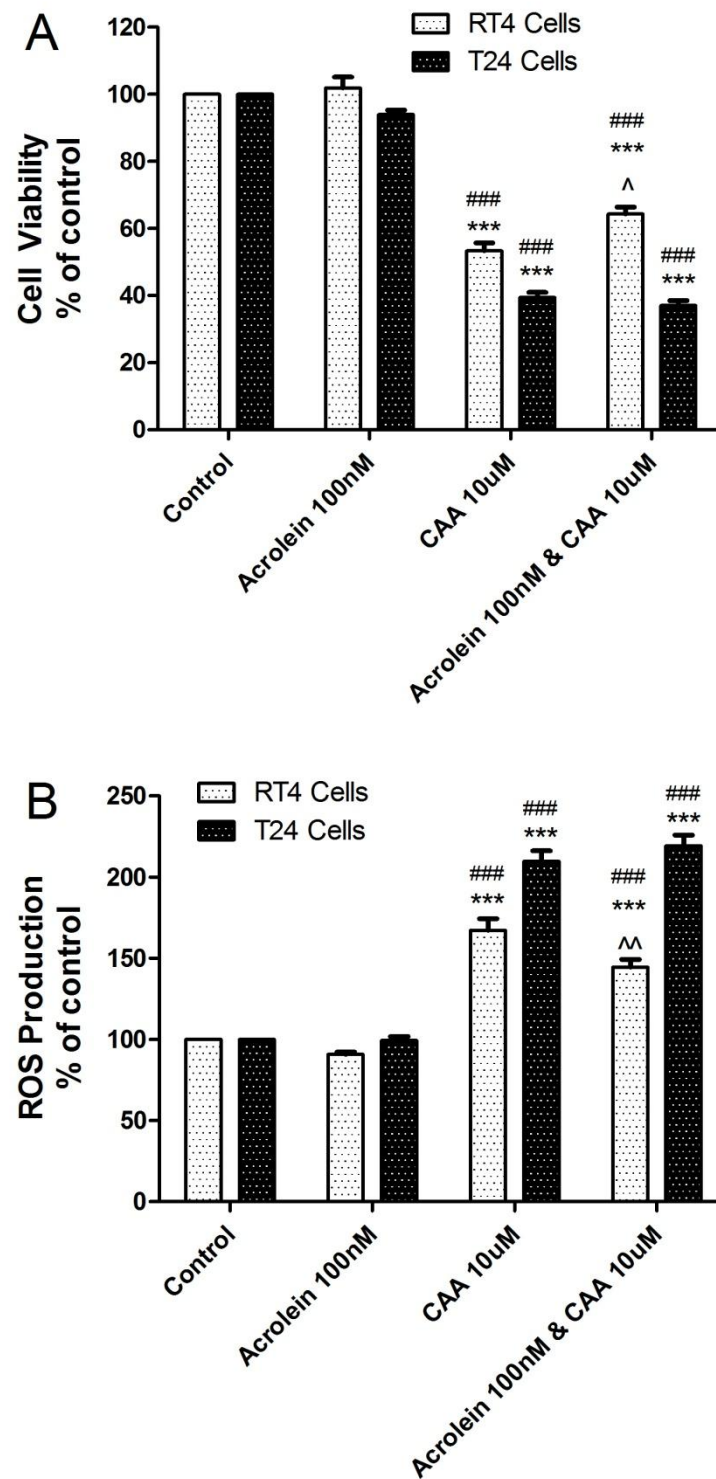


Figure 3.8: Effect of 24 hour acrolein (100nM), chloroacetaldehyde (CAA)(10μM) or acrolein (100nM) combined with CAA (10μM) treatment on cell viability (A) or reactive oxygen species (ROS) (B) in T24 and RT4 cells. Data are shown as a percentage of control (mean \pm SEM, n=6) and analysed by 1-way ANOVA with Tukey post-test. ^{###} p<0.001 vs control, ^{***}p<0.001 vs acrolein (100nM), [^]p<0.05 & ^{^^}p<0.01 vs CAA (10μM).

Table 3.2: Cell Viability and ROS formation in RT4 & T24 cells after treatment with CPO, IFO, Acrolein or CAA or a Combination								
		CPO	IFO	Acrolein		CAA		Combination
		100µM	100µM	10µM	100µM	10µM	100µM	100nM Acrolein + 10µM CAA
Cell Viability after treatment (% of control)	RT4	97.3±5.3	96.1±5.7	88.9±5.3	39.8±6.7**	50.4±2.9**	13.9±5.5**	64.3±2.0***
	T24	89.3±2.9	111±5.6	68.4±4.6**	1.63±1.6**	37.8±1.5**	8.60±4.8**	35.6±1.5***
ROS Formation after treatment (% of control)	RT4	87.6±7.2	91.6±6.5	102±5.8	264±47**	167±12**	509±120**	145±4.8***
	T24	105±4	84.6±6.9	128±8.2**	N/A	281±13.5**	N/A	219±6.4***
*p<0.05, **p<0.01, ***p<0.001 vs control								

Effect of Acrolein and CAA on Autophagy & Caspase-3 Activation

Acrolein and CAA both cause urothelial cell death and the pathway by which this happens was investigated next. Staining of autophagic vesicles using the Cyto-ID[®] Autophagy detection kit demonstrated that both RT4 and T24 cell lines have vesicles present in control baseline conditions as shown by the green fluorescence in **Figure 3.9** and **Figure 3.10** with the nucleus visualised by blue fluorescence. In particular the RT4 cell line has particularly high baseline autophagic activity (**Figure 3.9**). Treatment with acrolein (100 μ M) appears to slightly increase autophagic vesicles with staining appearing more intense and more diffuse within the cells. Treatment with CAA (10 μ M) had a greater effect than acrolein increasing autophagic vesicle formation with more intense staining appearing more tightly confined within the cells. The combination of acrolein (100nM) and CAA (10 μ M) produced a similar staining result as CAA (10 μ M) treatment alone.

In T24 cells, the baseline autophagy staining was much less than in the RT4 cells (**Figure 3.10**). Treatment with acrolein (100 μ M) had little effect on the appearance of autophagic vesicles, whereas CAA (10 μ M) treatment caused vesicle staining to increase and intensify (as shown by arrows in **Figure 3.10-C**). Treatment with the combination of acrolein (100nM) and CAA (10 μ M) produced a similar staining result as CAA (10 μ M) treatment alone.

Inhibition of autophagy with 3-Methyladenine (3MA) (5mM) caused decreased survival by 35 \pm 11% in untreated RT4 ($p < 0.05$) and 23 \pm 3% in T24 cells ($p < 0.01$). Cell death induced by acrolein (100 μ M), CAA (10 μ M) or the combination of acrolein (100nM) with CAA (10 μ M) was not significantly affected by co-treatment with 3MA (5mM) however there was a slight trend towards further cell death (**Figure 3.11**).

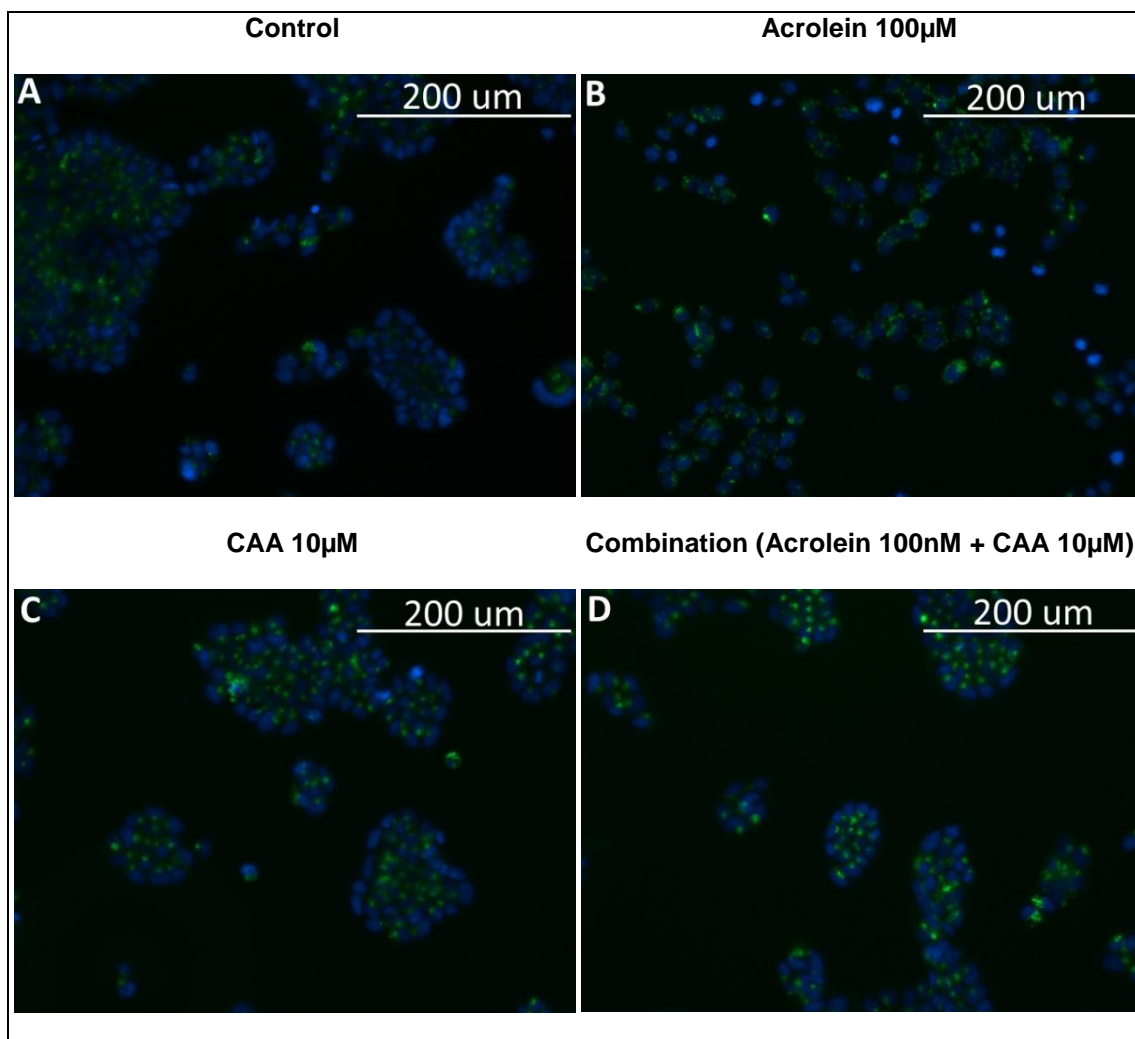


Figure 3.9: Staining of RT4 cells for autophagic vacuoles in green and nucleus in blue in control (A), acrolein (100μM) (B), CAA (10μM) (C), or a combination of acrolein (100nM) with CAA (10μM) (D) treated cells. Images are representative of 5 fields of view from n=3 experiments.

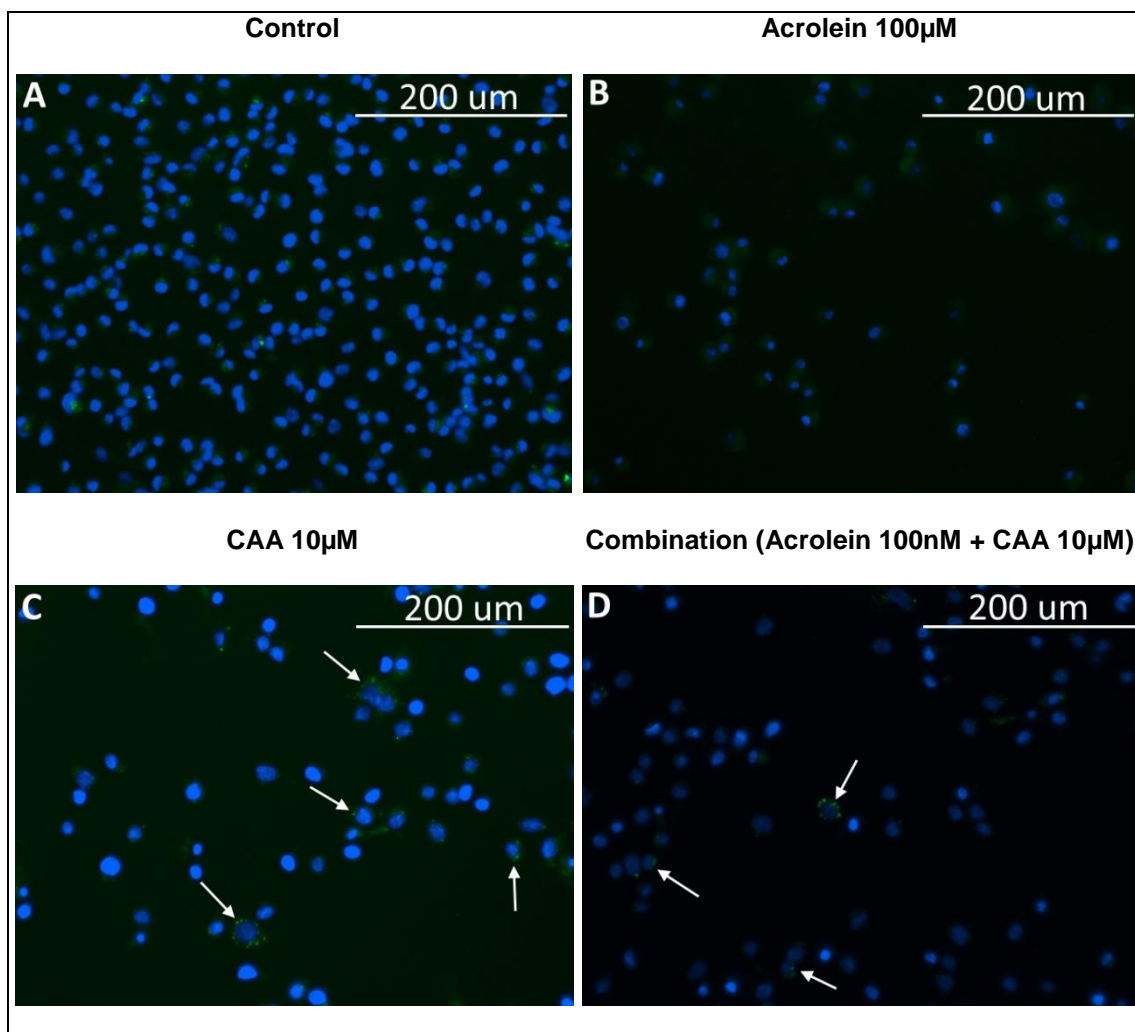


Figure 3.10: Staining of T24 cells for autophagic vesicles in green and nucleus in blue in Control and Acrolein, CAA or Combination treated cells. Images are representative of 5 fields of view from n=3 experiments.

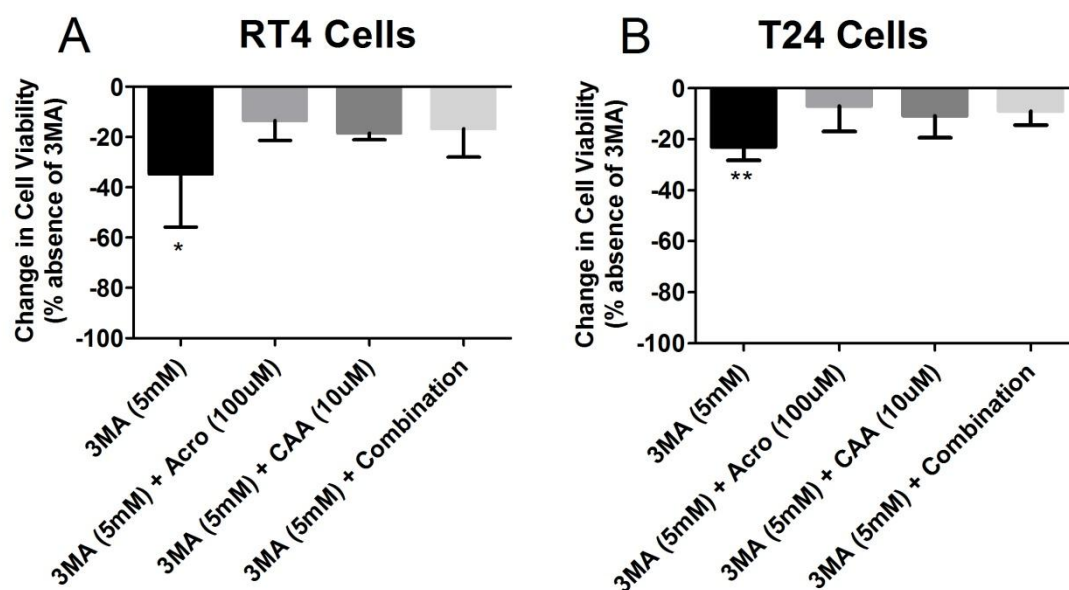


Figure 3.11: Effect of 3-Methyladenine (3MA) (5mM) on cell viability in untreated, acrolein (Acro) (100µM), CAA (10µM) or combination (Acrolein [100nM] + CAA [10µM]) treated RT4 (A) and T24 (B) cells. Data are expressed as a percentage change in viability induced by 3MA compared to the viability of matched treatments in the absence of 3MA. Mean \pm SEM (≥ 3) are shown and analysed using unpaired t-test between a treatment condition in the presence and absence of 3MA (* $p < 0.05$, ** $p < 0.01$ vs absence of 3MA).

The activity of the apoptosis protease, caspase-3, was significantly enhanced by CAA (10 μ M) treatment in both RT4 and T24 cells (2.5- and 3.5-fold respectively, $p < 0.01$) (**Figure 3.12**). Acrolein (100 μ M) increased caspase-3 in both cells lines although only significantly in T24 cells (by 2.5-fold, $p < 0.05$). The combination of acrolein (100nM) and CAA (10 μ M) caused increased caspase-3 activity in both cell lines (by approximately 2.5-fold in both cell lines, $p < 0.05$).

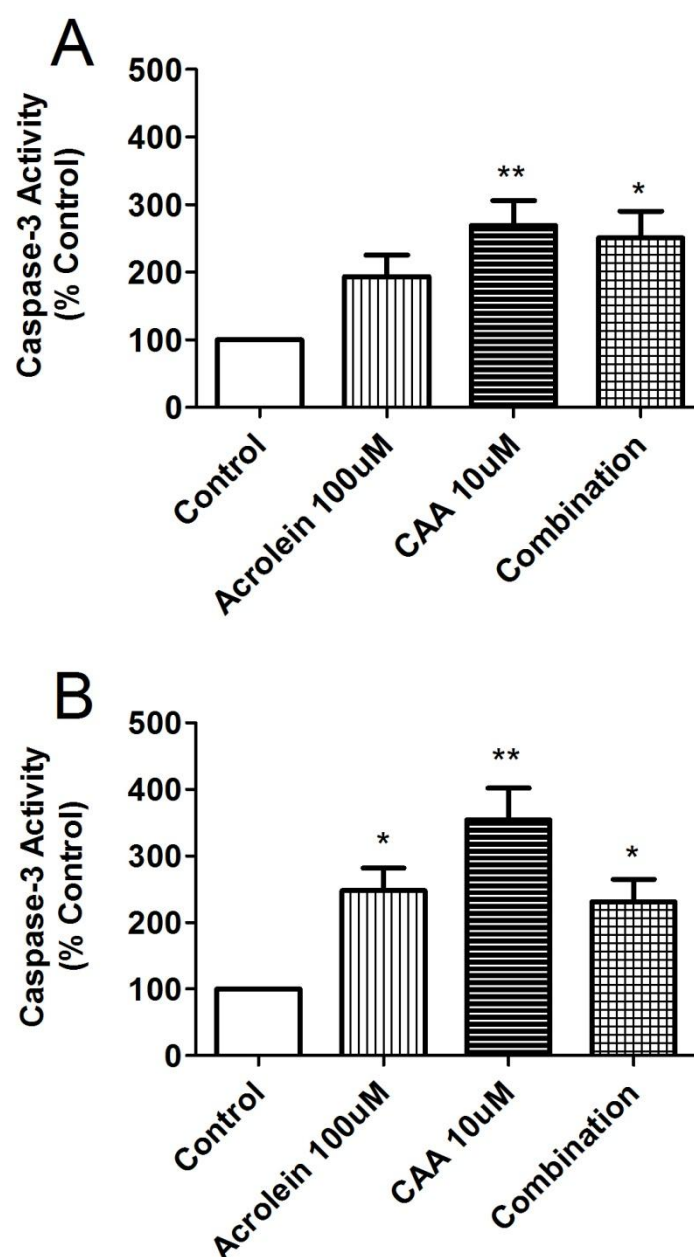


Figure 3.12: Effect of acrolein (100 μ M), CAA (10 μ M) or combination (Acrolein [100nM] + CAA [10 μ M]) treatment on caspase-3 activity in RT4 (A) and T24 (B) cells. Data represented as mean \pm SEM (≥ 3) and analysed using 1-way ANOVA with Tukey post-test (* $p < 0.05$, ** $p < 0.01$ vs control).

Effects of CPO, IFO, Acrolein and CAA on Urothelial Mediator Release

Given the importance of urothelial mediators in bladder function, the release of ATP, ACh and PGE₂ from human urothelial cells was investigated. Both RT4 and T24 cells released ATP in basal control conditions with concentrations at 15 minutes being 41.9 ± 2.9 nM and 97.9 ± 6.9 nM respectively (**Figure 3.13-A**). The levels of ATP increased significantly in response to 15 minute hypo-osmotic stimulation in both cell lines to 155 ± 14 nM and 141 ± 12 nM respectively ($p < 0.01$). Similarly, both cell lines released ACh in basal control conditions (**Figure 3.13-B**). In RT4 cells hypo-osmotic stimulation caused ACh levels to increase significantly from 0.99 ± 0.10 μ M to 2.83 ± 0.19 μ M ($p < 0.001$). While in T24 cells, ACh levels were significantly less after hypotonic stimulation (1.99 ± 0.14 μ M to 1.31 ± 0.10 μ M) ($p < 0.001$). Similar levels of PGE₂ were observed in basal and hypo-osmotic stretch conditions in RT4 cells (15.6 ± 1.3 and 21.8 ± 3.3 pg/ml respectively) and T24 cells (42.7 ± 7.0 and 34.6 ± 6.2 pg/ml respectively) (**Figure 3.13-C**).

Twenty four hour exposure of either cell line to 0.01-100 μ M cyclophosphamide or ifosfamide did not affect mediator release (**Figure 3.14** and **Figure 3.15** respectively). Even at the highest concentration (100 μ M) tested basal and stimulated levels of ATP, ACh and PGE₂ were similar to controls in both cell lines.

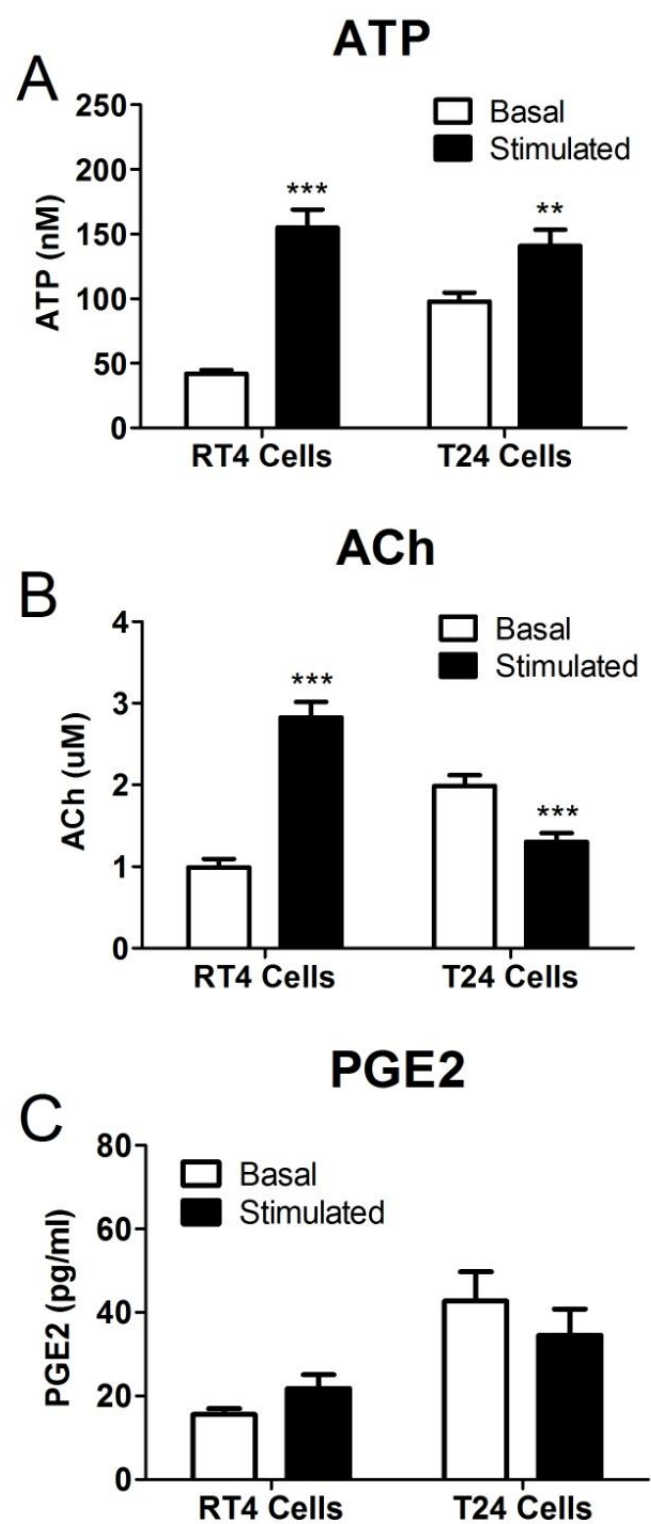


Figure 3.13: Basal and hypo-osmotically stimulated ATP (A), ACh (B) and PGE₂ mediator levels in RT4 and T24 cells. Data represented as mean \pm SEM (n \geq 22) and analysed by unpaired two-tailed t-test (** p<0.01, *** p<0.001, vs basal).

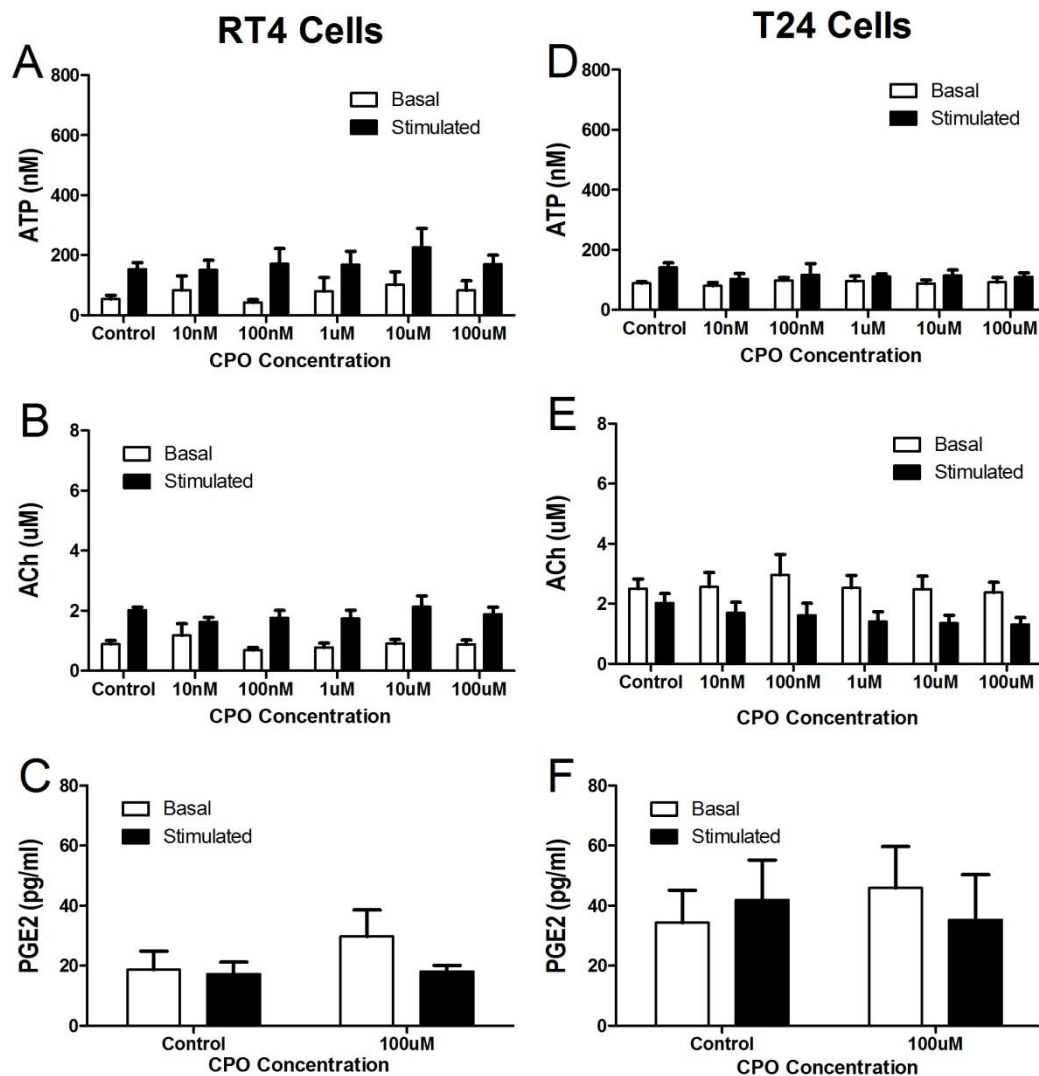


Figure 3.14: Effect of 24 hour cyclophosphamide (CPO) (10nM – 100μM) treatment on basal and hypo-osmotically stimulated ATP, ACh and PGE2 levels in RT4 (A, B & C respectively) and T24 cells (D, E & F respectively). Data are shown as mean ± SEM (n≥3).

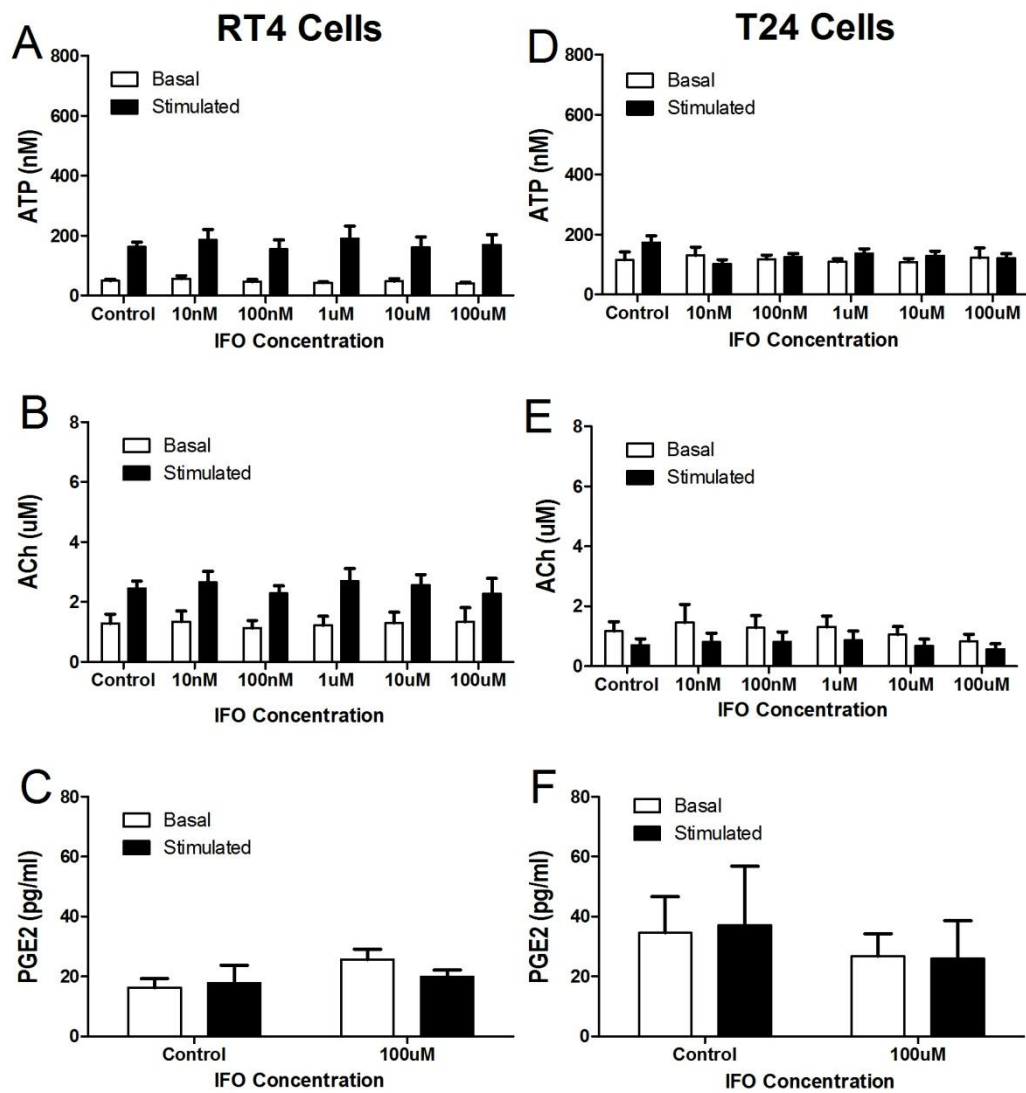


Figure 3.15: Effect of 24 hour ifosfamide (IFO) (10nM – 100µM) treatment on basal and hypo-osmotically stimulated ATP, ACh and PGE2 levels in RT4 (A, B & C respectively) and T24 cells (D, E & F respectively). Data are shown as mean \pm SEM ($n \geq 3$).

At 10 μ M, acrolein had no effect on ATP, ACh and PGE₂ levels in RT4 or T24 cells (**Figure 3.17**). However, CAA (10 μ M) in T24 cells, caused a significant increase in basal and stimulated ATP levels (3.5-fold increase in basal samples and 5-fold increase in stimulated samples [$p < 0.001$]) (**Figure 3.16-A**). Basal levels of ACh were significantly reduced while stimulated levels were significantly increased after treatment with 10 μ M chloroacetaldehyde (50% decrease in basal levels [$p < 0.05$] and 2.5-fold increase in stimulated levels [$p < 0.01$]) (**Figure 3.16-B**). In both cell lines, basal PGE₂ levels were increased significantly (approximately 2.5-fold increase in both cell lines [$p < 0.05$]) after chloroacetaldehyde treatment (10 μ M) (**Figure 3.16-C**). No significant change in ATP or ACh release was seen in RT4 cells after CAA (10 μ M) treatment (**Figure 3.16-D, E & F**).

At 10-fold higher concentrations, acrolein (100 μ M) treatment of RT4 cells did lead to changes in mediator release. Acrolein (100 μ M) caused a 5-fold increase in basal ATP levels ($p < 0.001$), 2.5-fold increase in stimulated ATP levels ($p < 0.05$) and a 60% increase in stimulated acetylcholine levels ($p < 0.001$) (**Figure 3.17-A & B**). PGE₂ levels in basal and stimulated samples from RT4 cells were also increased after treatment with 100 μ M acrolein (7.5- and 4-fold respectively [$p < 0.001$]) (**Figure 3.17-C**). No changes were observed in samples from T24 cells treated with 100 μ M acrolein as cell survival was too low.

Treatment of RT4 or T24 urothelial cells with acrolein (100nM) and CAA (10 μ M) in binary combination resulted in the same level of ATP, ACh and PGE₂ release as treatment with CAA alone (**Figure 3.18**). Specifically, in RT4 cells, the combination treatment did not change levels of ATP or ACh but did cause a 2.5-fold increase in basal PGE₂ levels ($p < 0.05$).

In T24 cells, basal and stimulated PGE₂ levels were also increased after combination treatment (5.4-fold and 3.3-fold respectively $p < 0.01$). Basal and stimulated ATP levels were increased by 2.2-fold ($p < 0.05$) and 4.2-fold respectively ($p < 0.001$) after combination treatment. Basal ACh levels were reduced by 50% ($p < 0.05$) while stimulated levels were increased 3-fold ($p < 0.05$) after acrolein (100nM) and CAA (10 μ M) combined treatment.

The absence of lactate dehydrogenase (LDH) in the extracellular fluid after treatment with acrolein or CAA and following the Krebs mediator collection procedure confirmed that mediators in the samples were released by the cells and were not simply leaking from damaged cells.

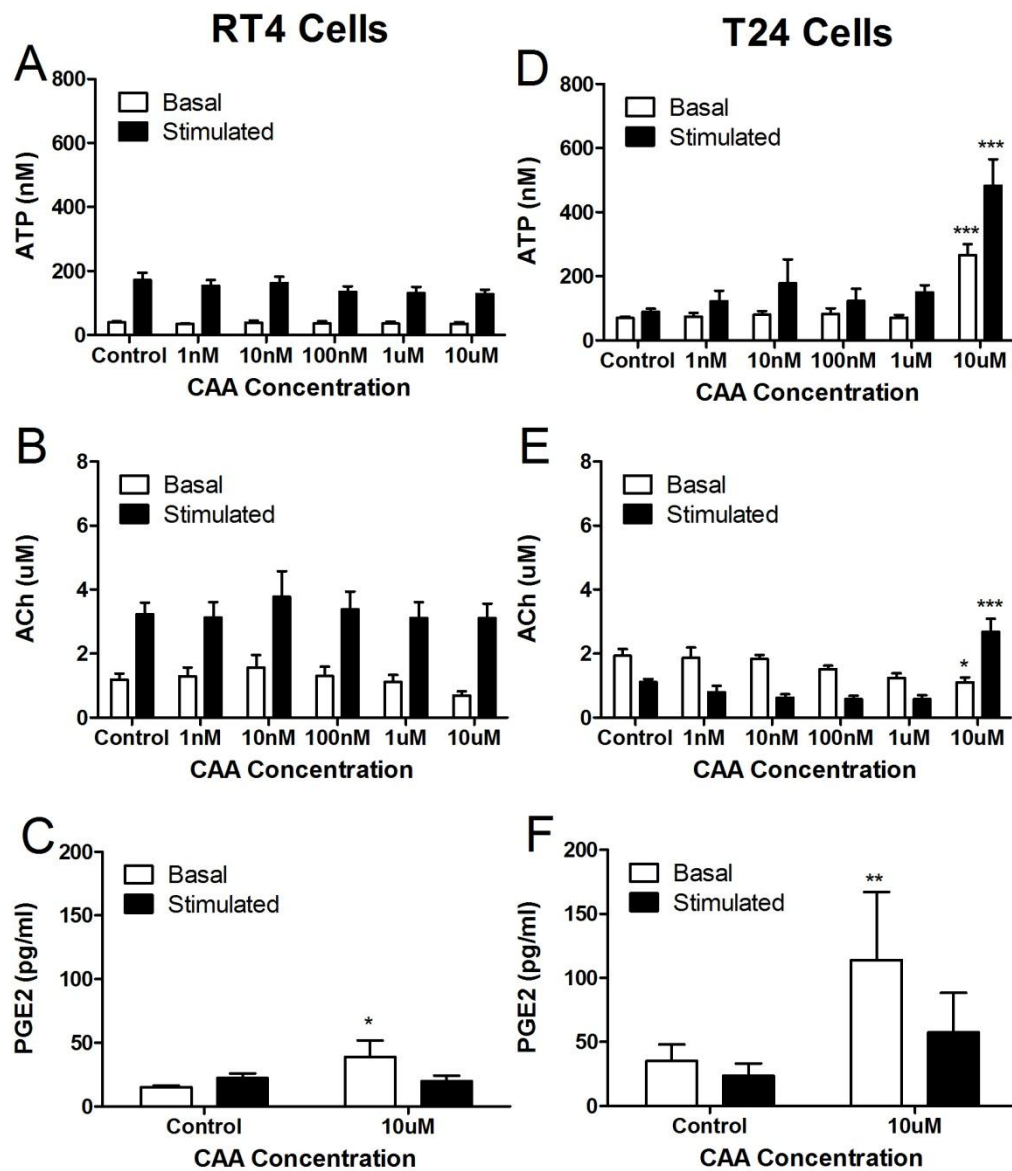


Figure 3.16: Effect of 24 hour chloroacetaldehyde (CAA) treatment on basal and hypo-osmotically stimulated ATP, ACh and PGE2 levels in RT4 (A, B & C respectively) and T24 cells (D, E & F respectively). Data are shown as mean \pm SEM (n=6) and analysed using 1-way ANOVA with Dunnett post-test or Students t-test. (* p<0.05, ** p<0.01, *** p<0.001, vs control).

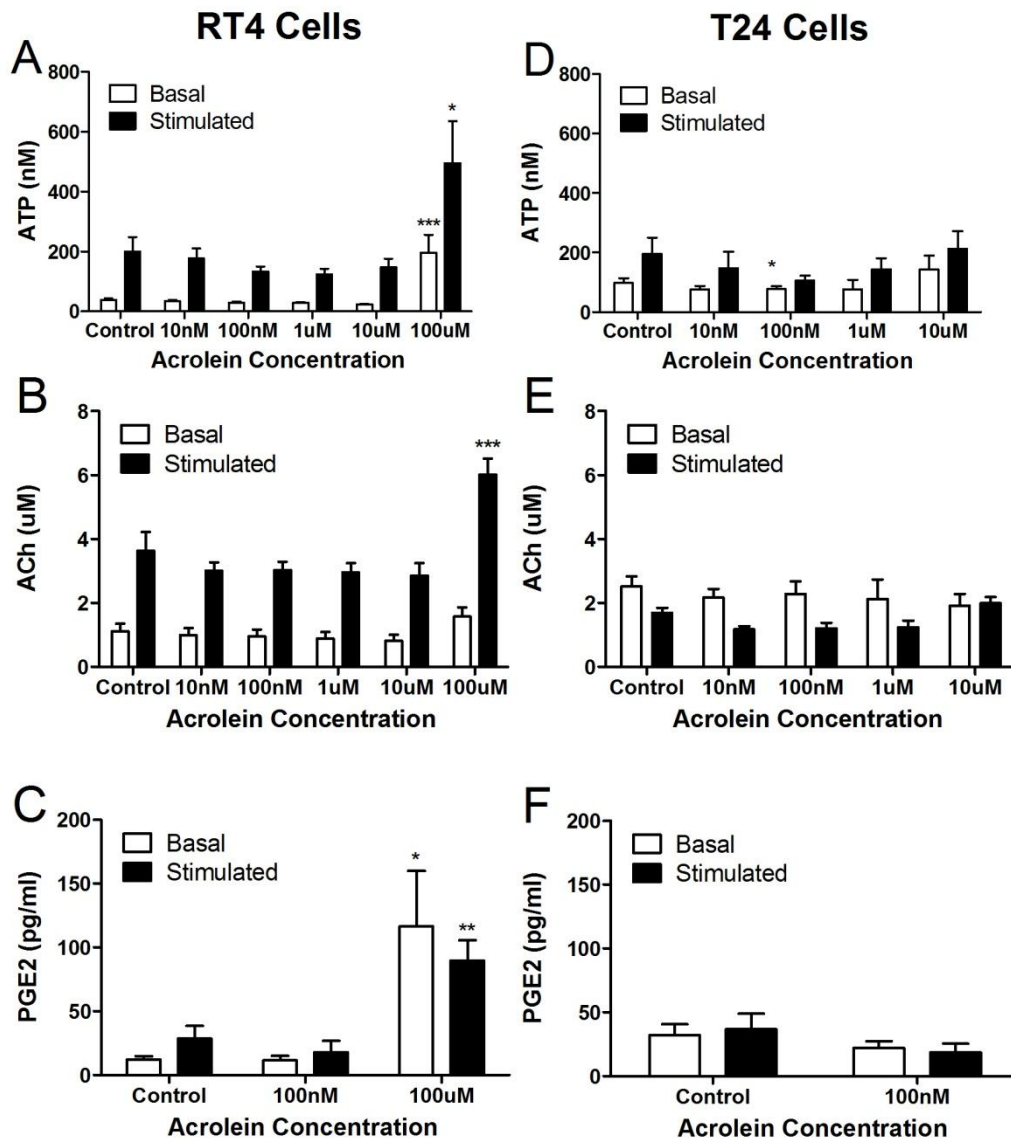


Figure 3.17: Effect of 24 hour acrolein treatment on basal and hypo-osmotically stimulated ATP, ACh and PGE2 levels in RT4 (A, B & C respectively) and T24 cells (D, E & F respectively). Data are shown as mean \pm SEM (n=6) and analysed using 1-way ANOVA with Dunnett post-test (* p<0.05, ** p<0.01, *** p<0.001, vs control).

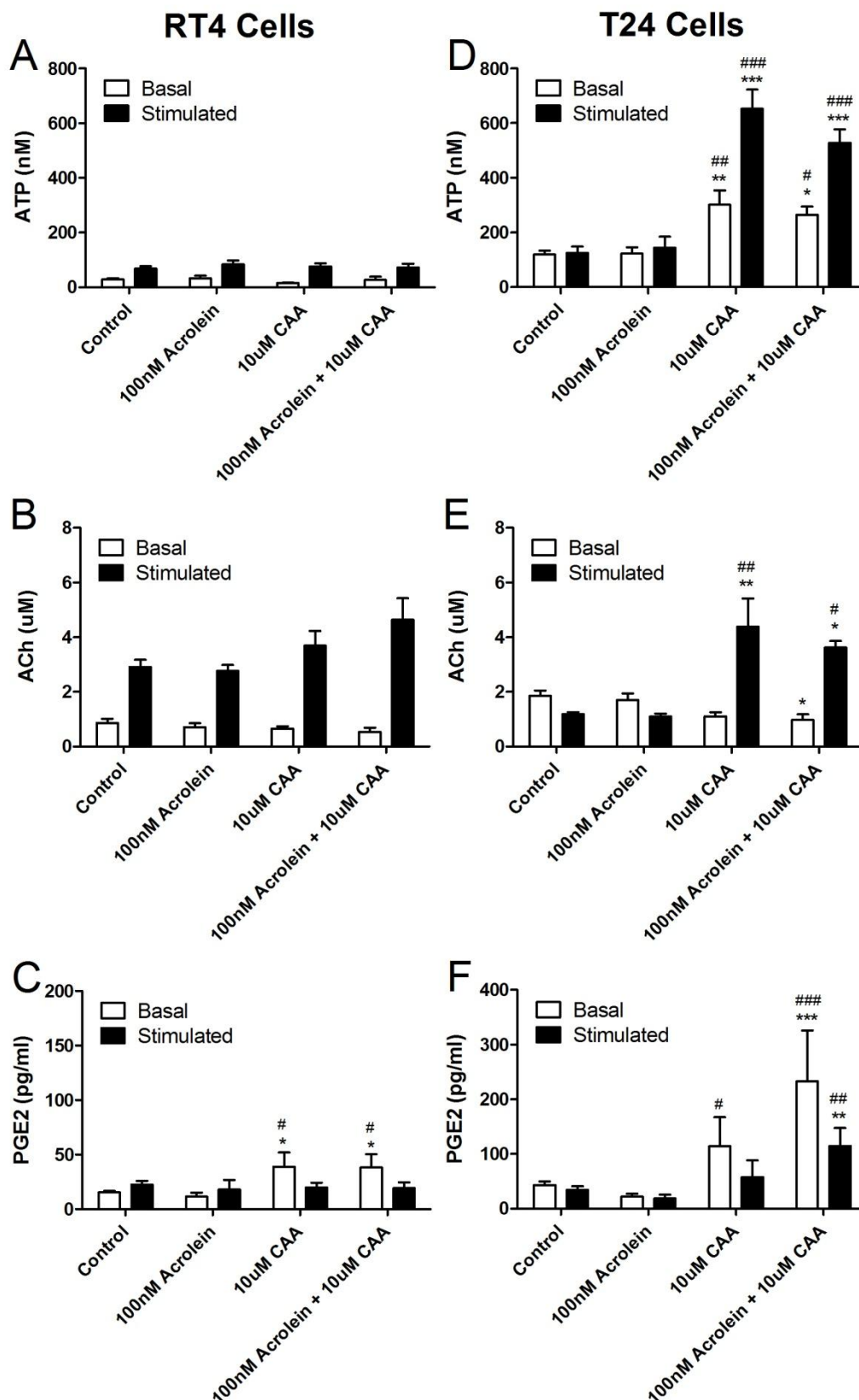


Figure 3.18: Effect of 24 hour acrolein (100nM), chloroacetaldehyde (CAA)(10μM) or acrolein (100nM) combined with CAA (10μM) treatment on basal and hypo-osmotically stimulated ATP, ACh and PGE2 levels in RT4 (A, B & C respectively) and T24 cells (D, E & F respectively). Data are shown as mean \pm SEM (n \geq 6) and analysed using 1-way ANOVA with Dunnett post-test (*p<0.05, **p<0.01, ***p<0.001, vs control & #p<0.05, ##p<0.01, ###p<0.001 vs acrolein).

Effects of CPO, IFO, Acrolein and CAA on Inflammatory Cytokine Release

To determine if inflammation plays a role in the urologic side effects of CPO and IFO, inflammatory cytokines were assessed. Specifically, levels of IL-8, IL-1 β , IL-6, IL-10, IL-12p70 and TNF in extracellular medium were measured after 24 hour CPO (100 μ M), IFO (100 μ M), acrolein (100 μ M) or CAA (10 μ M) treatment of RT4 and T24 cells.

Treatment of urothelial cells with CPO, IFO or CAA had no effect on release of the abovementioned cytokines whereas acrolein (100 μ M) treatment caused a number of them to increase. Specifically, in RT4 cells, IL-8 levels increased by 20-fold (**Figure 3.19-A**). IL-8 levels were above the readable range in samples from T24 cells however, IL-6 levels were increased 2.5-fold after acrolein (100 μ M) treatment ($p<0.01$). Furthermore, IL-1 β was undetected in T24 control samples but was detected after treatment with acrolein (100 μ M) ($p<0.001$) (**Figure 3.19-B**).

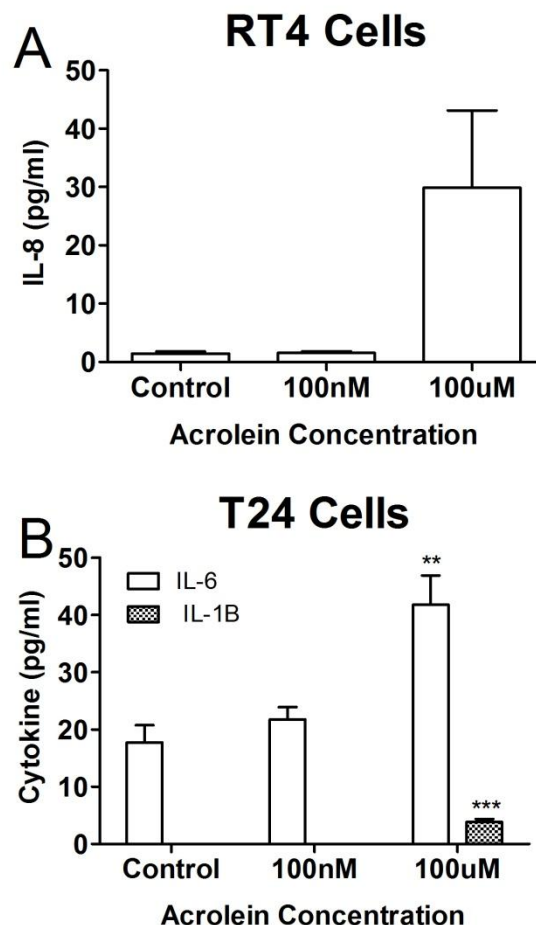


Figure 3.19: Effect of 24 hour acrolein treatment on cytokine levels from RT4 (A) and T24 (B) cells. Data are shown as mean \pm SEM ($n=3$) and analysed using 1-way ANOVA with Dunnett post-test (** $p<0.01$, *** $p<0.001$, vs control).

Effects of Vitamin C and N-acetylcysteine on Acrolein and CAA Induced Toxicity

Protecting the bladder from the damaging effect of acrolein and CAA would greatly improve the quality of life for patients treated with CPO or IFO. Accordingly, the potential protective effect of vitamin C and N-acetylcysteine (NAC) on acrolein and CAA induced urothelial cell damage was explored. Firstly, the effects of vitamin C and NAC alone were determined by treating the human urothelial cells with either substance (30 μ M – 3mM) for 24 hours and measuring cell viability and ROS production. The purpose of this was to determine optimal, non-toxic concentrations to use in further experiments. It was not relevant to the study to compare the effects of vitamin C and NAC alone. In RT4 cells vitamin C (3mM) reduced cell survival by 26.7 \pm 3.1% (p <0.001) and increased ROS production by 27.7 \pm 0.6% (p <0.001) (**Figure 3.20-A & C**). In T24 cells vitamin C (1mM and 3mM) was even more toxic causing cell survival to decrease by 21.2 \pm 5.5% and 61.2 \pm 3.0% respectively (p <0.05) and ROS production to increase by 18.9 \pm 1.6% and 96.6 \pm 4.5% respectively (p <0.001) (**Figure 3.20-B & D**). In RT4 cells NAC (1mM & 3mM) reduced cell survival by 14.6 \pm 3.5% and 25.9 \pm 5.3% respectively (p <0.01) with a subsequent increase in ROS production of 16.2 \pm 0.7% and 34.8 \pm 1.1% (p <0.001) (**Figure 3.20-A & C**). In T24 cells NAC (3mM) treatment had no effect on cell viability or ROS production.

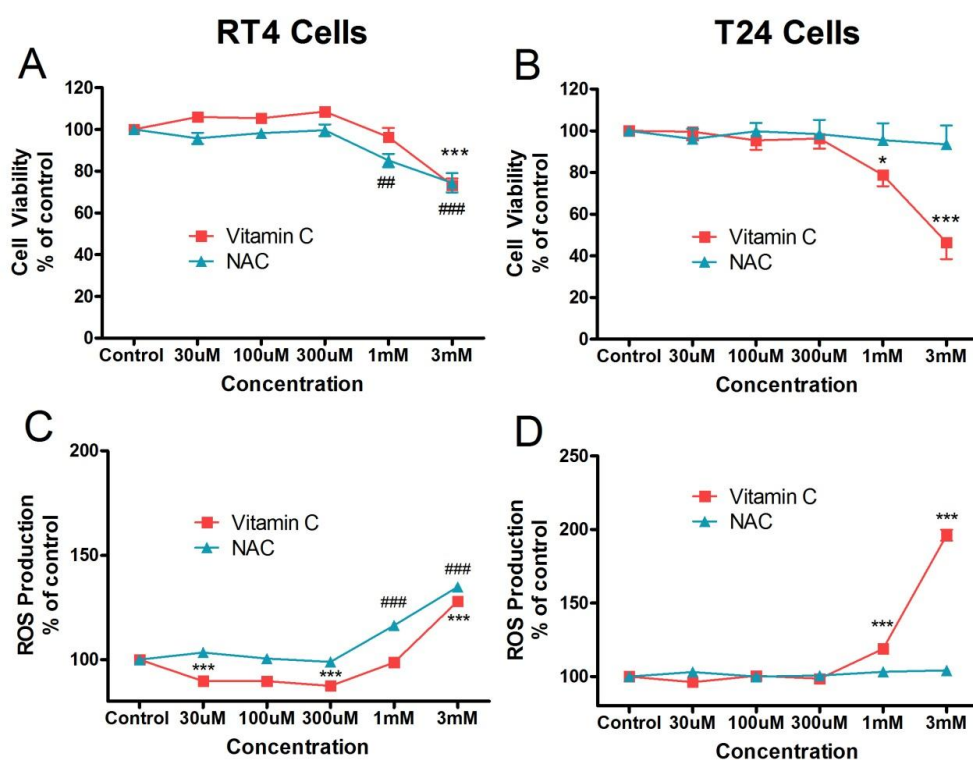


Figure 3.20: The effect of Vitamin C and N-acetylcysteine (NAC) on cell viability (A & B) and reactive oxygen species (ROS) production (C & D) in RT4 and T24 cells. Data are shown as mean \pm SEM (n=6) and analysed using 1-way ANOVA with Dunnett post-test (* p <0.05, ** p <0.01, *** p <0.001, vs Vitamin C control, ## p <0.01, ### p <0.001 vs NAC control).

Both vitamin C and NAC are acidic and the pH of McCoy's 5A culture medium containing vitamin C and NAC is shown in **Table 3.3**. The higher concentrations of 1mM and 3mM of vitamin C and NAC appeared to affect the pH of the culture medium. Accordingly, the effect of the pH change on RT4 and T24 cells was investigated. The reduction in pH had no effect on T24 cell survival or ROS production (**Figure 3.21-B & D**). However at a pH of 7.6 and 7.2, RT4 cell survival was reduced by $11.0 \pm 2.6\%$ and $14.3 \pm 3.1\%$ respectively ($p < 0.05$) (**Figure 3.21-A**). ROS production was increased at a pH of 7.2 by $11.9 \pm 3.3\%$ ($p < 0.01$) (**Figure 3.21-C**). Given the effects of vitamin C and NAC at high concentrations, further experiments were conducted using the optimal concentration of 300 μ M. A concentration of 1mM was also used as it only had modest damaging effects.

Table 3.3: pH of McCoy's 5A Culture Medium containing various concentrations of vitamin C or N-acetylcysteine (NAC)		
Concentration	Vitamin C	NAC
0	7.90	7.90
30uM	7.86	7.88
100uM	7.83	7.85
300uM	7.80	7.81
1mM	7.65	7.68
3mM	7.2	7.25

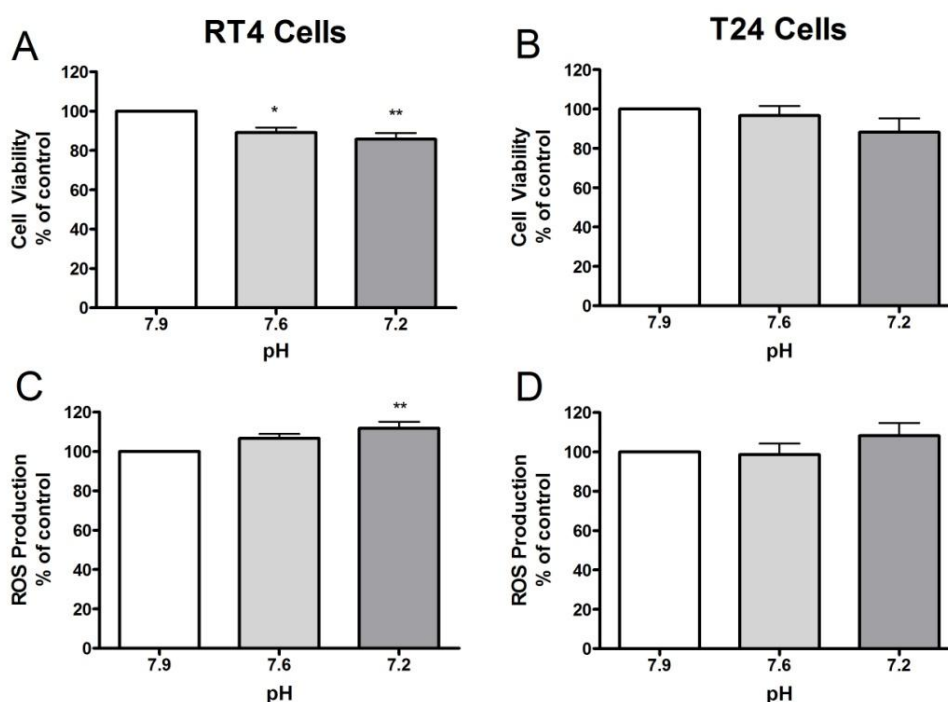


Figure 3.21: Effect of pH on cell viability (A & B) and reactive oxygen species (ROS) production in RT4 and T24 cells. Data are shown as mean \pm SEM (n=6) and analysed using 1-way ANOVA with Dunnett post-test (* $p < 0.05$, ** $p < 0.01$, vs control).

Acrolein (100 μ M) reduced cell survival to 37.2 \pm 2.4% in RT4 cells and 6.1 \pm 1.1% in T24 cells. The reduction in cell survival due to acrolein (100 μ M) was attenuated in the presence of NAC (300 μ M) in both RT4 and T24 cells restoring cell viability to 83.7 \pm 5.2% and 84.9 \pm 6.6% of control respectively (p <0.01) (**Figure 3.22–A & B**). Vitamin C (300 μ M) had no protective effects against acrolein (100 μ M) induced cell death with cell viability being similar to that seen after acrolein alone (26.4 \pm 3.9% and 0.8 \pm 1.3% of control in RT4 and T24 cells respectively) (**Figure 3.23-A & B**).

Similarly, CAA (10 μ M) reduced cell survival in RT4 cells to 62.0 \pm 2.8% of control and in T24 cells to 34.7 \pm 2%. Again, cell survival was restored to that of an untreated control by NAC (300 μ M) in RT4 (99.5 \pm 4.2% of control) and T24 cells (98.7 \pm 5.0%) (p <0.01) (**Figure 3.22 – C & D**). CAA (10 μ M) toxicity was not prevented by vitamin C (300 μ M) with viability being 62.8 \pm 2.6% in RT4 cells and 37.0 \pm 2.7% in T24 cells (**Figure 3.23 – C & D**).

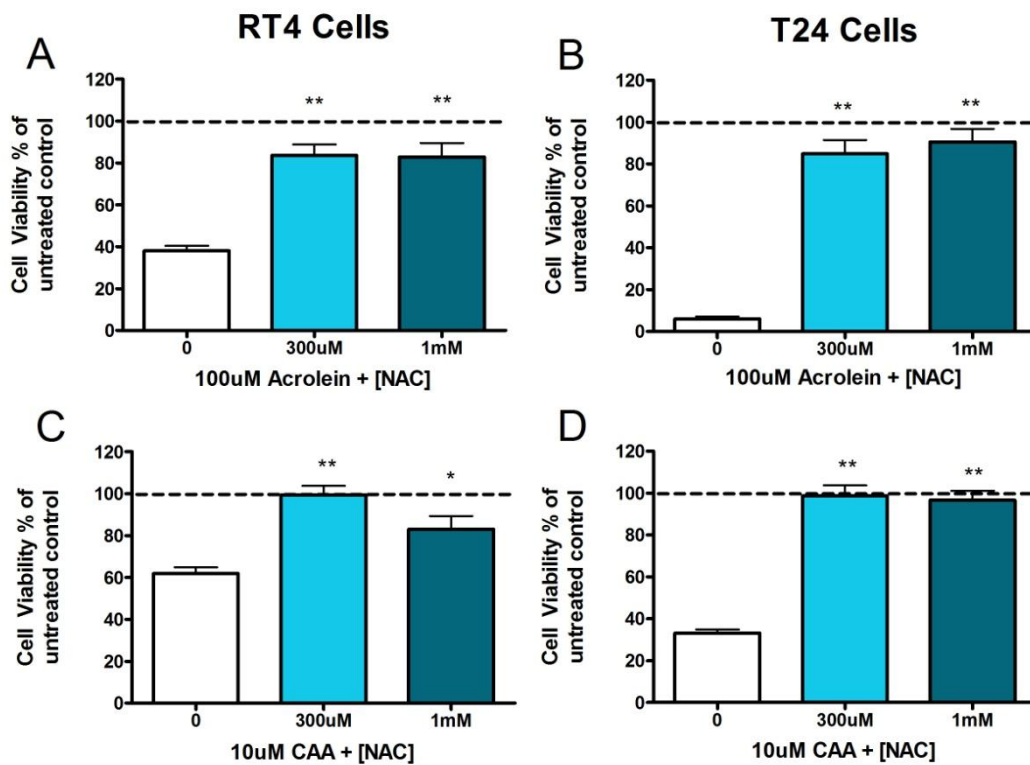


Figure 3.22: Protective effect of N-acetylcysteine (NAC) on acrolein (100 μ M) and CAA (10 μ M) induced cell death in RT4 (A & C respectively) and T24 cells (B & D respectively). Data represented as mean \pm SEM (n=6) and analysed via 1-way ANOVA with Dunnett post test (* p <0.05, ** p <0.01 vs absence of NAC).

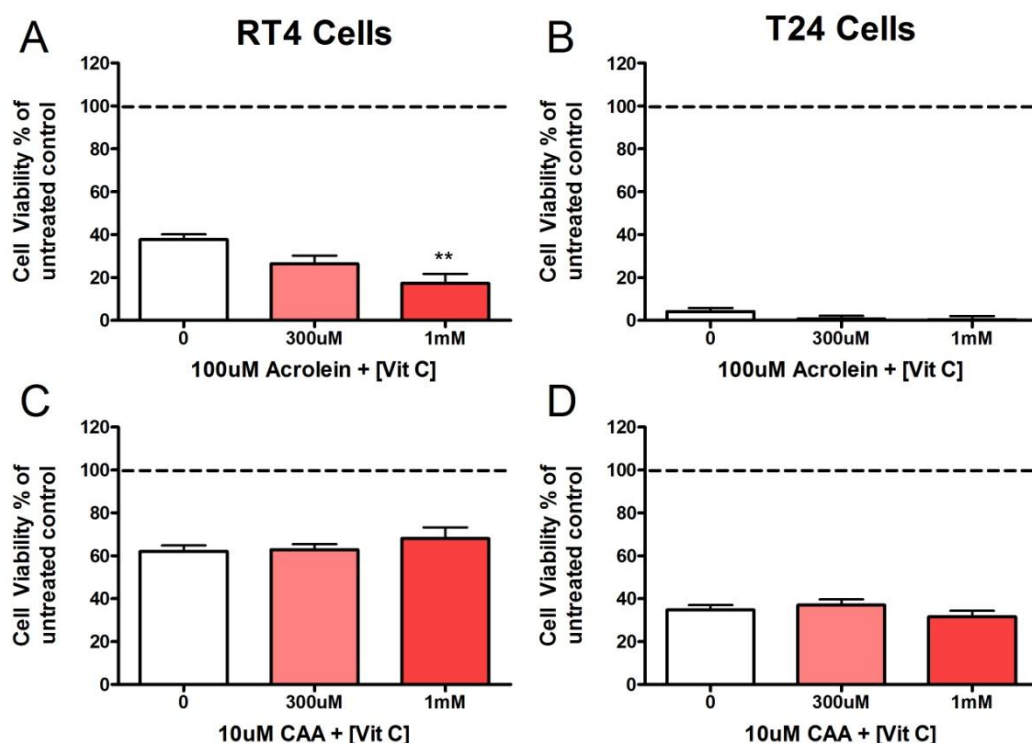


Figure 3.23: Effect of Vitamin C on acrolein (100 μ M) and CAA (10 μ M) induced cell death in RT4 (A & C respectively) and T24 cells (B & D respectively). Data represented as mean \pm SEM (n=6) and analysed via 1-way ANOVA with Dunnett post-test (*p<0.05, **p<0.01 vs absence of Vitamin C).

Acrolein (100 μ M) treatment caused basal ROS levels to increase to 305 \pm 8.9% of control levels in RT4 cells and to 1313 \pm 26.7% in T24 cells. NAC (300 μ M) prevented the increase in ROS production induced by acrolein in both cell lines, reducing ROS levels down to 116 \pm 3.5% of control in RT4 cells and 115.3 \pm 1.5% in T24 cells (p<0.01) (**Figure 3.24 – A & B**). Vitamin C (300 μ M) did not protect cells from acrolein induced ROS formation and rather had a pro-oxidant effect causing greater ROS levels in RT4 (650 \pm 32.5% of control) and T24 cells (8993 \pm 201% of control) (p<0.01) (**Figure 3.25 – A & B**).

Similarly, CAA increased ROS production to 242 \pm 16.6% and 272 \pm 4.7% of control in RT4 and T24 cells respectively. Again, ROS production was restored to that of an untreated control by NAC (300 μ M) (103 \pm 5.8% in RT4 and 101 \pm 2.0% in T24 cells) (p<0.01) (**Figure 3.24 – C & D**). Whereas, vitamin C had no protective effects and again had a pro-oxidant effect leading to a further increase in ROS formation to 199 \pm 19.7% and 299 \pm 6.4% in RT4 and T24 cells respectively (**Figure 3.25– C & D**).

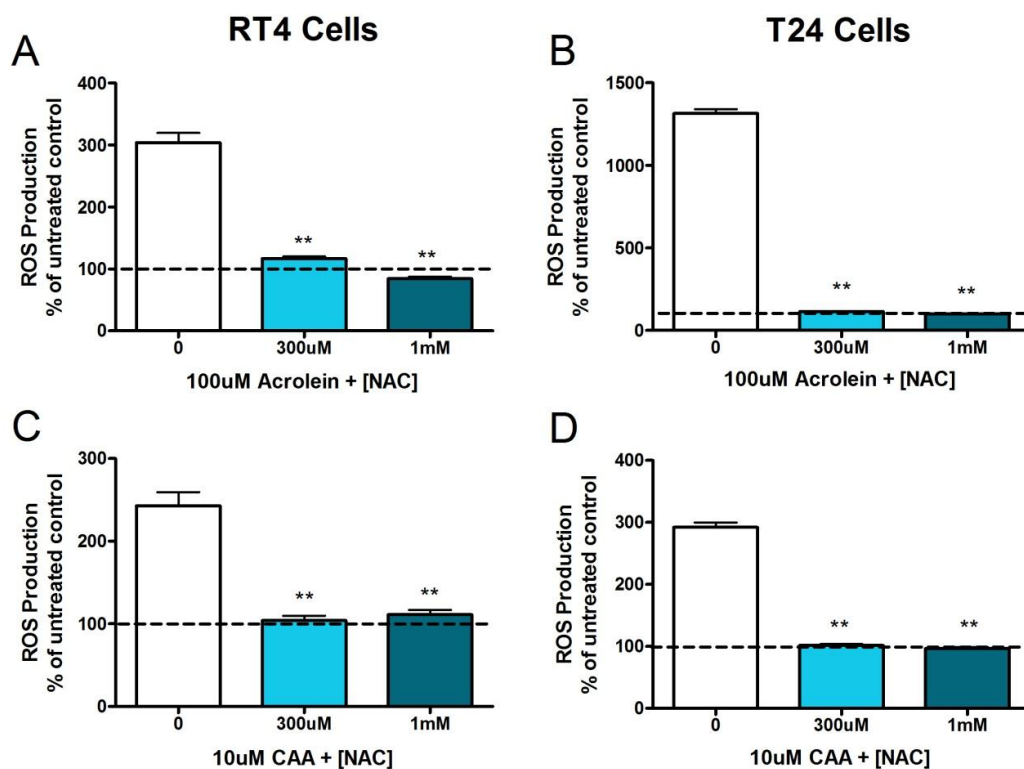


Figure 3.24: Protective effect of N-acetylcysteine (NAC) on acrolein (100 μ M) and CAA (10 μ M) induced reactive oxygen species (ROS) formation in RT4 (A & C respectively) and T24 cells (B & D respectively). Data represented as mean \pm SEM (n=6) and analysed via 1-way ANOVA with Dunnett post test (**p<0.01 vs absence of NAC).

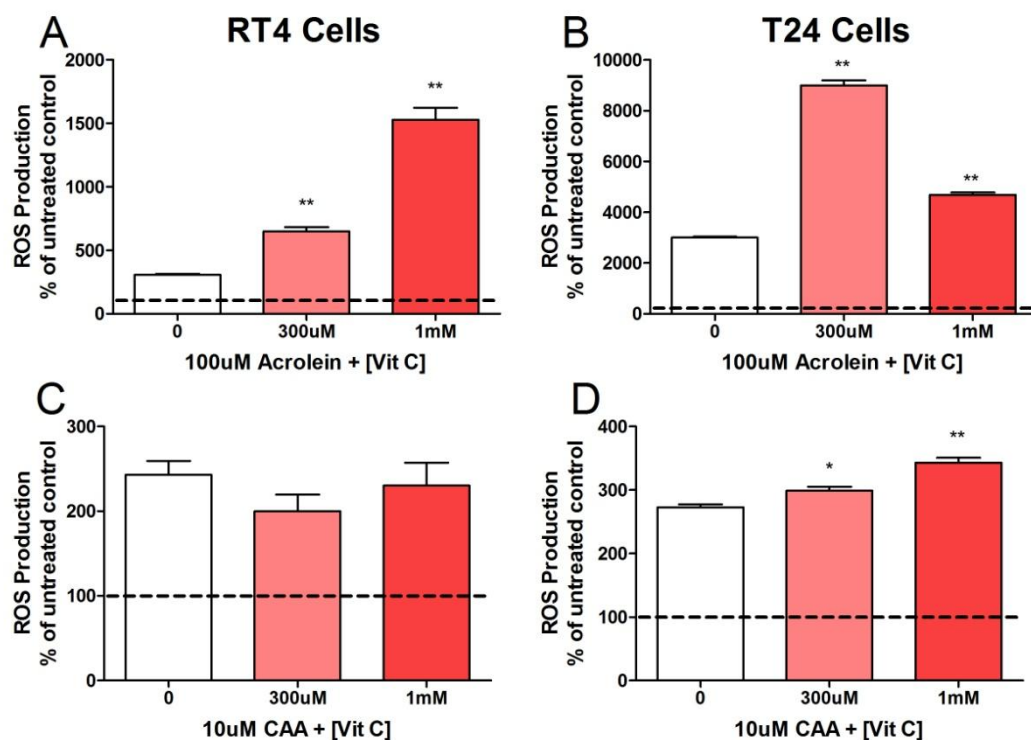


Figure 3.25: Effect of Vitamin C on acrolein (100 μ M) and CAA (10 μ M) induced reactive oxygen species (ROS) formation in RT4 (A & C respectively) and T24 cells (B & D respectively). Data represented as mean \pm SEM (n=6) and analysed via 1-way ANOVA with Dunnett post test (**p<0.01 vs absence of Vitamin C).

NAC also protected RT4 and T24 cells from the increases in caspase-3 activity induced by acrolein (100 μ M), CAA (10 μ M) and the combination of acrolein (100nM) and CAA (10 μ M). Co-treatment with NAC (300 μ M) protected urothelial cells from CAA (10 μ M), reducing caspase-3 levels from 269 \pm 37% down to 83.4 \pm 8.8% of control in RT4 cells and from 355 \pm 48% to 179 \pm 37% of control in T24 cells (p<0.05) (**Figure 3.26**). The acrolein (100 μ M) induced increase in caspase-3 activity in T24 cells was also prevented by NAC (300 μ M) with levels decreasing from 248 \pm 34% to 130 \pm 18% of control (p<0.05). NAC (300 μ M) also protected urothelial cells from increased caspase-3 activity due to the combination of acrolein (100nM) with CAA (10 μ M) reducing levels from 251 \pm 39% to 106 \pm 16% of control in RT4 cells and from 323 \pm 33% to 91.5 \pm 20% in T24 cells (p<0.05) (**Figure 3.26**).

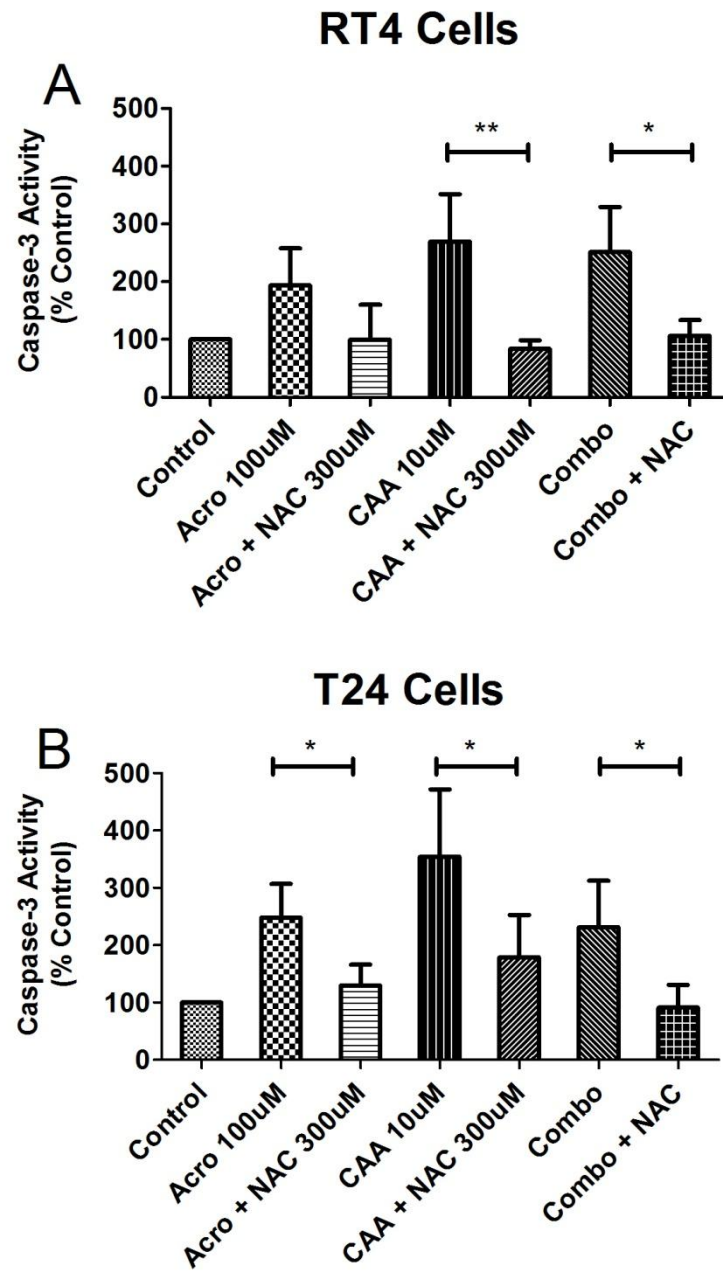


Figure 3.26: Effect of N-acetylcysteine (NAC) (300 μ M) on the increase in Caspase-3 activity induced by acrolein (100 μ M), CAA (10 μ M) or combination (Acrolein [100nM] + CAA [10 μ M]) treatment in RT4 (A) and T24 (B) cells. Data represented as mean \pm SEM (≥ 3) and analysed using unpaired t-test between treatments in the presence and absence of NAC (* $p < 0.05$, ** $p < 0.01$ vs absence of NAC).

Effects of Vitamin C and N-acetylcysteine on Urothelial Mediator Release Changes After Acrolein and CAA Treatment

NAC can protect human urothelial cells from cell death and ROS production induced by acrolein and CAA, but its protective effects on the functional changes to mediator release was considered next. Acrolein (100 μ M) treatment in RT4 cells caused a significant increase in both basal and stimulated ATP, PGE₂ and ACh levels. These changes in mediator release could be prevented by co-treatment with NAC (300 μ M) (**Figure 3.27**). Specifically, the change in basal and stimulated ATP levels due to acrolein (100 μ M) (177 \pm 32nM and 409 \pm 95nM respectively [p<0.05]) was prevented by the addition of NAC (300 μ M) producing ATP levels (18.4 \pm 8.3nM and 70.6 \pm 20.6nM respectively [p<0.05]) comparable to that of the untreated control (24.9 \pm 6.1nM and 73.3 \pm 12.9nM respectively) (**Figure 3.27-A**). Similarly, basal and stimulated ACh release was increased after acrolein (100 μ M) treatment (1.78 \pm 0.23 μ M and 6.56 \pm 0.61 μ M respectively [p<0.05]) and this could be prevented by NAC (300 μ M) returning levels (0.83 \pm 0.06 μ M and 3.43 \pm 0.58 μ M respectively [p<0.05]) close to that of the control (1.02 \pm 0.14 μ M and 2.69 \pm 0.24 μ M respectively) (**Figure 3.27-B**). Basal and stimulated levels of PGE₂ were also enhanced after acrolein (100 μ M) treatment (212 \pm 56pg/mL and 94 \pm 23pg/mL respectively [p<0.001]) but again this change could be prevented in the presence of NAC (300 μ M) again maintaining levels (17.4 \pm 4.1pg/mL and 30.0 \pm 5.9pg/mL respectively [p<0.05]) similar to control (17.3 \pm 1.8pg/mL and 21.2 \pm 3.9pg/mL respectively) (**Figure 3.27-C**).

In T24 cells CAA (10 μ M) treatment caused a significant increase in both basal and stimulated ATP and PGE₂ levels. Basal levels of ACh were reduced while stimulated levels were increased after CAA (10 μ M) treatment. Co-treatment with NAC (300 μ M) could prevent these changes in mediator release (**Figure 3.28**). Specifically, the change in basal and stimulated ATP levels due to CAA (10 μ M) (304 \pm 32nM and 596 \pm 94nM [p<0.01]) was prevented by the addition of NAC (300 μ M) producing ATP levels (107 \pm 13nM and 228 \pm 56nM [p<0.01]) comparable to that of the untreated control (124 \pm 20nM and 163 \pm 40nM) (**Figure 3.28-A**). In the presence of CAA (10 μ M) and NAC (300 μ M) the lowered basal ACh levels (1.24 \pm 0.12 μ M [p<0.01]) were returned to 1.96 \pm 0.13 μ M which was similar to control levels (1.95 \pm 0.15 μ M). Similarly, the increased stimulated ACh levels (2.62 \pm 0.25 μ M) due to CAA (10 μ M) were also prevented by NAC (300 μ M) returning levels to 2.04 \pm 0.10 μ M, close to that of controls (1.33 \pm 0.12 μ M) (**Figure 3.28-B**). Lastly, the increased basal and stimulated PGE₂ levels seen after CAA (10 μ M) treatment (109 \pm 41pg/mL and 90 \pm 25pg/mL [p<0.05]) were prevented by NAC (300 μ M) treatment, with levels decreasing to 35.3 \pm 16.2pg/mL and 49.6 \pm 11.1pg/mL respectively, similar to control levels (41.8 \pm 6.8pg/mL and 38.9 \pm 6.0pg/mL) (**Figure 3.28-C**).

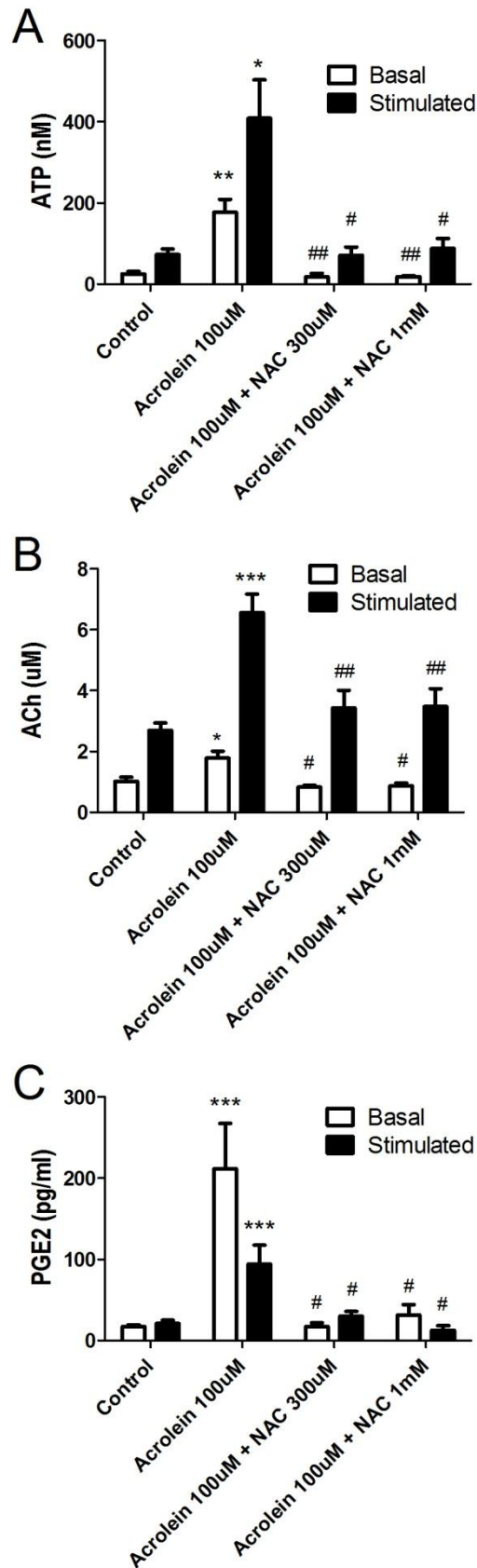


Figure 3.27: Effect of 24 hour acrolein (100µM) treatment in the absence and presence of N-acetylceysteine (NAC) (300µM & 1mM) on basal and hypo-osmotically stimulated ATP (A) acetylcholine (B) and PGE₂ (C) levels in RT4 cells. Data are shown as mean ± SEM (n≥4) and analysed using 1-way ANOVA with Tukey post-test (* p<0.05, ** p<0.01, *** p<0.001, vs control, #p<0.05, ##p<0.01, ###p<0.001, vs acrolein (100µM) alone).

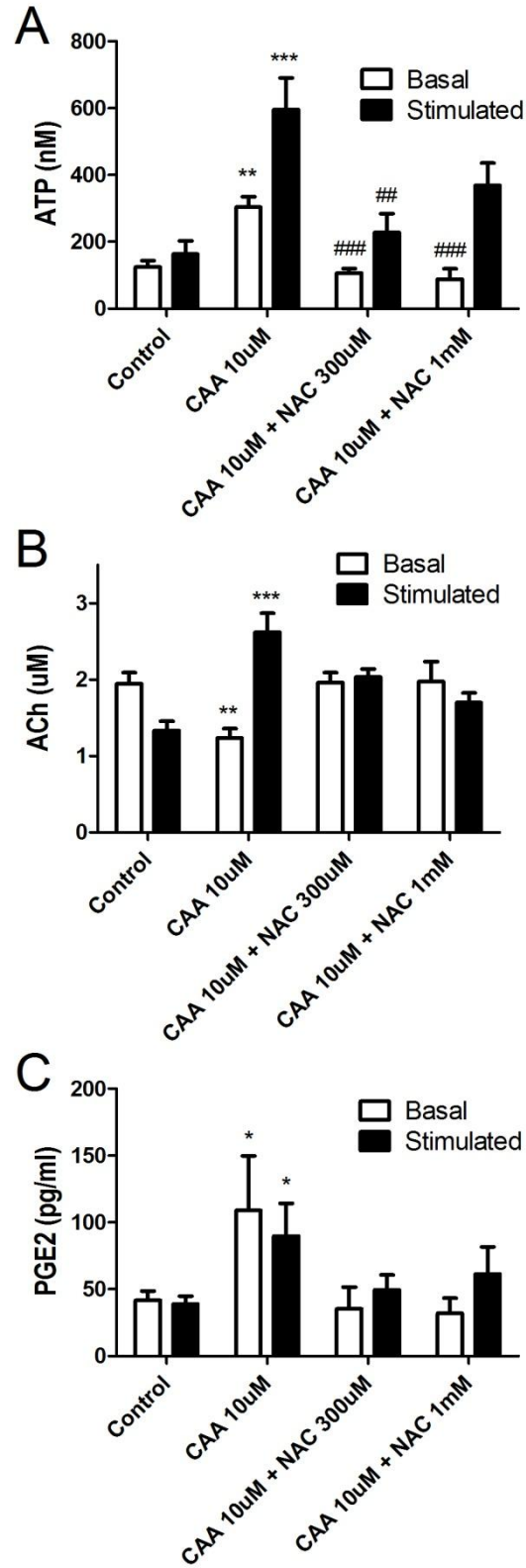


Figure 3.28: Effect of 24 hour chloroacetaldehyde (CAA) (10µM) treatment in the absence and presence of N-acetylcysteine (NAC) (300µM & 1mM) on basal and hypo-osmotically stimulated ATP (A) acetylcholine (B) and PGE₂ (C) levels in T24 cells. Data are shown as mean ± SEM (n≥4) and analysed using 1-way ANOVA with Tukey post-test (* p<0.05, ** p<0.01, *** p<0.001, vs control, ## p<0.01, ### p<0.001, vs CAA(10µM) alone).

3.4 DISCUSSION

The urothelium has been shown to be able to communicate with the detrusor, myofibroblasts and sensory nerves by releasing ATP, ACh and PGE₂ among other mediators (Birder et al., 2010b). Changes in these transmitters have been linked to bladder function diseases and sensory changes (Kumar et al., 2010, Chen et al., 2003, Sun and Chai, 2006, Sun et al., 2001).

Cyclophosphamide and ifosfamide are known to cause acute haemorrhagic cystitis and lasting urotoxic symptoms such as increased frequency of micturition, feelings of incomplete emptying, pain and dysuria. The results in this study demonstrated that high concentrations of both CPO and IFO did not affect the survival of human urothelial cells lines. This effect was expected and has been observed previously in similar studies. For example, CPO or IFO did not cause cell damage or death of human proximal tubule cells at concentrations up to 300µM (Schwerdt et al., 2006). However, more importantly this study investigated urothelial cell function not just cell death and treatment with CPO or IFO had no effect on the release of mediators from the urothelial cells. These results support the understanding that the parent drugs are not responsible for their urotoxicity and that it is the urotoxic metabolites that are responsible. However, almost exclusively the metabolite acrolein has been blamed for the bladder toxicity despite another toxic metabolite, CAA, being excreted in the urine. Therefore, the effect of both acrolein and CAA on human urothelial cells was investigated and compared.

Is Acrolein or CAA Toxic to Human Urothelial Cells?

The results in this study demonstrated that urothelial cell survival was reduced by acrolein and CAA, confirming that both metabolites are toxic to the urothelium. Firstly, both acrolein and CAA were more toxic to T24 cells, affecting cell survival and ROS formation at concentrations 10-fold lower, than in RT4 cells. Given what we know about the characteristics of these two cell lines this is not surprising. As discussed above and as shown in **Figure 3.1** the RT4 cells form tight connections between the cells growing in a layer similar to normal urothelium, whereas the T24 cells do not interact with each other and grow separately (Kabaso et al., 2011). The interaction between the urothelial cells is important for the barrier function of the urothelium and appears to protect cells from damage due to acrolein and CAA. As a model, the RT4 cells would be a better representation of normal or healthy urothelium and how it reacts to a single dose of CPO or IFO. After CPO treatment the urothelium undergoes an acute period of damage characterised by ulceration and erosion resulting in the loss of cell to cell interaction and epithelial barrier function (Auge et al., 2013, Juszczak et al., 2010). The surviving cells then start proliferating rapidly in order to repair the denuded areas (Boudes et al., 2011,

Golubeva et al., 2014, Romih et al., 2001). CPO and IFO are often given in a repeated dose regimen over days, weeks or months. Accordingly, the T24 cells may act as a model of the urothelial cells in a proliferative state with fewer cell to cell interactions than would be present during subsequent treatments, after initial injury has occurred.

It appears that chloroacetaldehyde is more toxic to urothelial cells than acrolein, affecting cell viability at a 10-fold lower concentration (10 μ M/1 μ M in RT4/T24 cells) than acrolein (100 μ M/10 μ M in RT4/T24 cells). Given that acrolein is largely blamed for the urotoxicity of CPO and IFO this result was unexpected. However, similar results have been reported in human renal epithelial cells wherein CAA was more potent than acrolein inducing apoptosis at lower concentrations (15-75 μ M) compared to acrolein (150-300 μ M) after 24 hours (Schwerdt et al., 2006).

Acrolein had no significant effect at concentrations below 100 μ M in RT4 cells while modest toxicity was observed at 10 μ M in T24 cells. Acrolein concentrations of 100nM have been reported in the urine of patients (Takamoto et al., 2004), so for toxicity to occur only at 100-1000-fold higher concentrations was unexpected. However, similar results have been seen in human bronchial epithelial cells where significant loss of cell viability was observed only at acrolein concentrations of 50 μ M or higher (Nardini et al., 2002). Additionally, in human keratinocytes, acrolein caused cell death at 50 μ M (Takeuchi et al., 2001). Therefore, this result appears to be comparable with other epithelial cells.

The reduction in cell viability after treatment with acrolein was accompanied by an increase in ROS production. This supports the theory that acrolein damage in the bladder is due to ROS production (Korkmaz et al., 2007). Previous studies in bronchial epithelial cells have shown that acrolein (25 μ M) increases ROS production more than 5-fold after 6 hours exposure (Nardini et al., 2002). While several studies in rodents have demonstrated that antioxidants prevent CPO or IFO induced bladder damage including flavanoids (Ozcan et al., 2005), α -tocopherol, β -carotene and melatonin (Sadir et al., 2007) as well as GSH and amifostine (Batista et al., 2007).

CAA treatment at 100 μ M in RT4s and 10 μ M in T24s also caused a significant increase in ROS production. This not only supports that CAA and acrolein toxicity occurs by similar mechanisms but also supports a role for CAA in CPO and IFO induced urotoxicity. ROS production is thought to be an important mechanism in programmed necrosis by inducing lipid peroxidation or altering the function of certain channel proteins (Moquin and Chan, 2010). However, ROS can also regulate apoptosis under certain conditions. Whether ROS triggers apoptosis or necrosis appears to rely on factors such as the cellular ATP level and the concentration of ROS

produced (Hildeman et al., 1999, Miyoshi et al., 2006). Apoptosis will be considered in more detail below.

Interestingly, ROS production did not change after CAA treatment at lower concentrations (10 μ M in RT4s and 1 μ M in T24s) despite cell viability being significantly reduced. This suggests that CAA toxicity at higher concentrations may involve ROS, similar to acrolein toxicity, while at lower concentrations may induce an alternative cell death pathway. A study by Macallister et al., (2012) showed that depletion of the endogenous antioxidant GSH increased the toxicity of acrolein but not CAA in rat hepatocytes which also supports that CAA may cause cell death via a pathway other than ROS production. Furthermore, cell survival after acrolein and CAA treatment was investigated in normoxic (95% O₂) and hypoxic (1% O₂) conditions. Although cell survival was improved in the absence of oxygen it was still significantly less than control suggesting both CAA and acrolein toxicity occurs via a pathway other than just ROS production (Macallister et al., 2012).

However, both acrolein and CAA would be present in the urine together and given that both metabolites cause damage by similar mechanisms it is logical to assume there could be a greater effect when exposed to a combination. Surprisingly, combining the likely urinary concentrations of acrolein (100nM) and CAA (10 μ M) was not more toxic than CAA treatment alone. Rather, acrolein (100nM) appeared to have a slight antagonistic effect protecting against cell death and ROS formation when combined with CAA (10 μ M) in RT4 cells. At lower concentrations acrolein can affect transcription factors such as nuclear factor-kappaB (NF- κ B) (Horton et al., 1999) and activator protein-1 (AP-1) (Biswal et al., 2002, Korkmaz et al., 2007). Both of these transcription factors can cause cell injury or cell proliferation depending on the tissue. In gut epithelial cells, inhibition of NF- κ B activation caused cell death indicating a protective effect in these cells (Chen et al., 2003). In urothelial cells, induction of AP-1 transactivation caused increased cell proliferation (Simeonova et al., 2000). Therefore, it is possible that the lower concentration of acrolein (100nM), by way of transcription factors, may be protecting the cells from CAA induced death.

Twenty four hour treatment with CAA (10 μ M) enhanced caspase-3 activity in both cell lines. However, acrolein (100 μ M) only significantly enhanced caspase-3 activity in the T24 cell line and to a lesser extent than CAA. This again suggests that CAA is more toxic to urothelial cells, inducing greater levels of apoptosis at a 10-fold lower concentration. Similar results have been reported in proximal tubule cells where caspase-3 activity was enhanced by CAA at much lower concentrations (15-75 μ M) than acrolein (150-300 μ M) after 24 hours (Schwerdt et al., 2006).

Caspase-3 activation is an important step in the apoptotic pathway and the enhanced levels seen after acrolein and CAA treatment supports the concept that these metabolites can cause urothelial cell death by apoptosis. Recently it has also been shown that ROS formation can cause apoptosis in T24 urothelial cells via the intrinsic pathway (Jo et al., 2014, Wang et al., 2013b). Furthermore, acrolein has also been shown to cause ROS mediated apoptosis in male germ cells (He et al., 2014), while CPO induced apoptosis of human granulosa cells has also been shown to involve ROS formation (Tsai-Turton et al., 2007). The results in this study support a similar mechanism of acrolein and CAA induced toxicity in human urothelial cells. In particular, given that the ROS scavenger NAC prevented both the increase in ROS production and the increase in caspase-3 activity it appears possible that the two mechanisms are linked. The protective role of NAC is discussed in more detail below.

The chemotherapeutic cisplatin has been shown to enhance apoptosis in T24 urothelial cells by increasing autophagic activity (Yang et al., 2013). Therefore, autophagy was investigated next as a possible contributor to acrolein and CAA induced urothelial cell death. Autophagy is important in cancer as it can prevent tumorigenesis or enable survival of established tumours (Amaravadi et al., 2011). As the cells used in this study were derived from human bladder cancers it was first necessary to establish whether autophagy was important for the cell survival. Inhibition of autophagy with 3-MA significantly reduced RT4 and T24 cell viability indicating that autophagy contributes to survival and/or proliferation in these cell lines. Treatment with acrolein and CAA appeared to increase staining for autophagic vacuoles in both cell lines. However, inhibiting autophagy during treatment with acrolein and CAA had no significant effect on cell survival and if anything caused a slight decrease in survival. This suggests that autophagy does not contribute to acrolein or CAA toxicity.

Does Acrolein or CAA Affect Urothelial Mediator Release?

Acrolein and CAA reduce urothelial cell viability but how these compounds affect the function of urothelial cells may be more important in terms of side effects experienced by patients. It is now well established that urothelial mediators can affect the function of the bladder. Firstly, urothelial ATP plays a major role in bladder sensory mechanotransduction mechanisms. Urothelial cells release ATP in basal or normal conditions and additional ATP release can be stimulated in response to stretch (Kang et al., 2013, Sadananda et al., 2009). Urothelial ATP acts on P2X3 and possibly P2X2 receptors on sensory nerves to transmit the sensation of bladder filling to the central nervous system (Burnstock, 2009). ATP can have effects on both low and high threshold nerve fibres, altering the micturition reflex and pain sensations

respectively (Burnstock, 2009, Rong et al., 2002). In this study acrolein and CAA increased both basal and stimulated ATP release from urothelial cells. More ATP release from the urothelium may lead to increased sensory nerve activity and sensations of a greater volume than is actually present in the bladder. Higher basal ATP levels in patients treated with cyclophosphamide or ifosfamide may be responsible for the feelings of incomplete emptying and the subsequent urinary frequency experienced by patients. An increase in stretch induced ATP levels after cyclophosphamide or ifosfamide treatment may also contribute to more frequent urges to urinate and could be responsible for the reported painful sensations as the bladder fills or during voiding.

It has also been reported that ATP increases the excitability of afferent nerves (Burnstock, 2011) and enhances its own release from urothelial cells (Birder, 2010), while cyclophosphamide treatment enhances and sensitises P2X receptor activity on nerves (Dang et al., 2008). Furthermore, a P2X3 and P2X2/3 antagonist has been shown to reduce the bladder hyperactivity of CPO treated rats (Ito et al., 2008). Studies of a comparable disease in cats (i.e. feline interstitial cystitis) have demonstrated increased stretch-evoked release of ATP from the urothelium as well as changes in purinergic receptor profiles in urothelial cells (Birder et al., 2003). Therefore, the combined effect of more ATP and more sensitive purinergic receptors on afferent nerves is a likely explanation of the sensory changes seen after cyclophosphamide and ifosfamide treatment. Both acrolein and CAA treatment resulted in an increase in ATP release from human urothelial cells which may contribute to the urotoxicity of cyclophosphamide and ifosfamide. However, CAA was more toxic than acrolein, inducing the changes in ATP at a 10-fold lower concentration (10 μ M) than acrolein potentially making CAA the more urotoxic metabolite.

The urothelium has also been shown to release ACh in response to stretch which is thought to act on muscarinic receptors on the urothelium and sensory nerves (Yoshida et al., 2006). The activation of urothelial muscarinic receptors leads to release of several substances including ATP and PGE₂ as well as UDIF (Hanna-Mitchell and Birder, 2008, Yokoyama et al., 2011). The affect of ACh on afferent nerves in the bladder is still unclear with both inhibitory and stimulatory effects reported (Daly et al., 2010, Iijima et al., 2007).

Acrolein and CAA treatment both caused increased stretch-induced levels of ACh, however CAA was again the more potent metabolite inducing this change at a concentration 10-fold lower (10 μ M) than acrolein (100 μ M). The increased ACh during stretch may be acting directly on the detrusor muscle or the afferent nerves to affect functional and sensory changes in the

bladder. Enhanced filling pressure has been reported in animals treated with CPO or IFO (Okinami et al., 2014, Macedo et al., 2008a, Wang et al., 2008b, Kageyama et al., 2008) and a possible explanation of this is excess ACh released from the urothelium causing increased tone of the detrusor. However, it is also possible that the change in ACh levels alters the release of other transmitters from the urothelium, indirectly contributing to the bladder changes after cyclophosphamide or ifosfamide treatment. Specifically, more ACh may act in an autocrine manner causing greater ATP or PGE₂ release which in turn could lead to enhanced detrusor tone or altered bladder sensations in patients.

The urothelium expresses COX-1 and COX-2 enzymes which are responsible for the synthesis of prostanoids including PGE₂ (de Jongh et al., 2009, de Jongh et al., 2007). The COX-1 enzyme is active in normal conditions and COX-2 is inducible suggesting that COX-1 is responsible for normal physiological synthesis of prostanoids and COX-2 is activated during inflammation (Tramontana et al., 2000). PGE₂ causes contraction of isolated human detrusor muscle (Andersson, 1993) however it appears that physiologically it is not directly involved in efferent control of the bladder and rather plays a role as a neuromodulator of afferent and efferent transmission (Andersson and Arner, 2004).

Several studies in rats and mice have found that systemic treatment with cyclophosphamide, ifosfamide or intravesicular treatment with acrolein induces COX-2 expression in urothelium within 12-24 hours of treatment (Macedo et al., 2011, Macedo et al., 2008b, Klinger et al., 2007) and one study found that the plasma PGE₂ concentration was increased after administration of ifosfamide (Macedo et al., 2011). However, the present study is the first to investigate the effect of acrolein and CAA on the release of PGE₂ from urothelial cells. Acrolein treatment (100µM) caused an increase in basal and stimulated PGE₂ release from the urothelial cells. CAA treatment (10µM) also caused significantly more basal PGE₂ to be released from urothelial cells and while stimulated release was increased it was not significant. PGE₂ released from the urothelium is thought to activate or sensitise afferent nerves (de Groat and Yoshimura, 2001) and cause stimulation of the detrusor muscle increasing resting tone and spontaneous contractions (de Jongh et al., 2007).

CPO treatment in rats reduced intercontraction interval by almost 40% and this was restored when co-treated with an EP4 receptor antagonists (Chuang et al., 2012). Mice developed bladder pain-like nociceptive behaviour and bladder inflammation after CPO treatment and preadministration of an EP1 receptor antagonist prevented the pain symptoms (Miki et al., 2011). These studies support a role for PGE₂ production in CPO and IFO induced detrusor

overactivity and pain symptoms. PGE₂ may be acting directly on detrusor or afferent nerves to cause urotoxic side effects in patients however, it may also be acting indirectly via the urothelium. For example, PGE₂, caused increased ATP release from the rat urothelium during both distended and non-distended conditions (Tanaka et al., 2011).

Both acrolein and CAA caused higher basal and stimulated PGE₂ levels in human urothelial cells. The increased PGE₂ may cause sensitisation of afferent nerves, increased ATP release from the urothelium and increase afferent activity as a result. This mechanism may explain the altered micturition reflexes, hyperactivity and abnormal or painful sensations experienced by patients treated with cyclophosphamide or ifosfamide. Again, CAA appeared to be more potent inducing changes in PGE₂ at a concentration (10µM) 10-fold lower than acrolein (100nM).

Does Acrolein or CAA Affect Urothelial Cytokine Release?

Various cytokines are thought to be produced by urothelial cells during cyclophosphamide or ifosfamide induced cystitis leading to inflammation (Gomes et al., 1995, Ribeiro et al., 2002). Inflammation has been shown to increase bladder afferent neurone excitability and is thought to contribute to bladder overactivity and pain (Hayashi et al., 2009, Wyndaele and De Wachter, 2003, Yoshimura et al., 2002).

Malley and Vizzard (2002), found that CPO treatment in rats caused a significant increase in the cytokines interleukin (IL)-1 β , IL-2, IL-4, IL-6, TNF- α and TNF- β and that their inhibition, particularly TNF- α and IL-1 β , reduced the urothelial erosion, haemorrhage, oedema, and leukocyte migration observed. In addition, cytokines IL-1 β , IL-6 and IL-17 were increased in the urine of rats treated with CPO and reducing their synthesis caused improved urodynamic parameters including micturition frequency, basal pressure and residual urine volume (Nasrin et al., 2013). Furthermore, intravesical instillation of acrolein in rats caused increased IL-6 production, iNOS expression and bladder haemorrhage while co-treatment with an IL-6 neutralising antibody reduced these effects (Wang et al., 2013a). These studies support the idea that inflammatory cytokines play a role in bladder overactivity and pain, however very few describe potential the mechanisms involved. It has been shown that cytokines can alter gap junctions in bladder smooth muscle and interstitial cells thereby affecting cell-to-cell communication (Heinrich et al., 2011). Furthermore, inflammation increased bladder afferent excitability in rats with cystitis by decreasing expression of Kv1.4 α -subunits of voltage-gated K⁺ channels (Hayashi et al., 2009).

The present study in human urothelial cells also found an increase in interleukin (IL)-1 β , IL-6 and IL-8 after treatment with acrolein (100 μ M), while CAA (10 μ M) did not affect the levels of cytokines measured. The induction of IL-1 β , IL-6 and IL-8 demonstrated here may therefore be another factor contributing to the bladder overactivity and pain experienced by patients following CPO or IFO treatment.

Does N-acetylcysteine or Vitamin C Protect Urothelial Cells From Acrolein and CAA Toxicity?

Acrolein and CAA are toxic to human urothelial cells and induce changes in urothelial mediator release that may explain or contribute to the symptoms experienced by patients after CPO or IFO treatment. Accordingly, to protect the urothelial cells from damage due to acrolein or CAA would be advantageous to patients, potentially preventing or reducing the adverse effects and even improving treatment tolerability and outcomes.

As discussed previously, acrolein toxicity can occur by its direct binding to proteins, DNA and GSH or indirectly via ROS production causing further GSH depletion, protein and DNA damage (Korkmaz et al., 2007) and CAA toxicity is thought to act via similar mechanisms (Sood and O'Brien, 1993).

The antioxidant vitamin C did not protect human urothelial cells from cell death or ROS production due to acrolein or CAA. This was unexpected given that vitamin C can react with acrolein through Michael addition (Zhu et al., 2011) and protects human bronchial epithelial cells from apoptosis due to acrolein (Nardini et al., 2002). However, a study in rats treated with CPO showed that leukocyte infiltration and oedema was only partially protected by oral administration of vitamin C while bladder haemorrhage was not prevented (Farshid et al., 2013). Furthermore, vitamin C was unable to protect human hepatocytes from acrolein or CAA toxicity and showed pro-oxidant effects at concentrations above 300 μ M (Macallister et al., 2012). In this study vitamin C also caused pro-oxidant effects at higher concentrations (300 μ M & 1mM). The ability of high concentrations of vitamin C to cause cell death has been described previously and its toxicity is thought to involve increased H₂O₂ production (reviewed by Ohno et al., 2009). Interestingly, the pro-oxidant effects of vitamin C appeared greater in combination with acrolein with milder effects seen in combination with CAA. This suggests a possible interaction between vitamin c and acrolein. However, it is thought that H₂O₂ detoxification in response to high dose vitamin C is performed by glutathione peroxidase using GSH in the reaction. Both acrolein and CAA are thought to deplete GSH due to direct binding or via production of reactive oxygen species and therefore should react similarly to increased

oxidative stress due to vitamin C. However, the cell viability after acrolein was much lower than that after CAA and therefore it is possible that the cells had greater GSH depletion and were more susceptible to the toxicity of vitamin C. Nonetheless, it appears that vitamin C may not be useful in protecting the urothelium from damage due to acrolein and CAA. Rather, the results in this study support a pro-oxidant role for vitamin C in urothelial cells.

NAC is an amine-protected version of cysteine. In the human body NAC is rapidly hydrolysed back to cysteine, which is the rate-limiting substrate for GSH synthesis (Sen, 2001, Gross et al., 1993). Cysteine can also react directly with acrolein itself and it is the preferential site of acrolein adduct formation in proteins (Gross et al., 1993, Zhu et al., 2011). In this study, NAC protected the urothelial cells from acrolein and CAA induced cell death and ROS formation restoring cell survival and ROS levels to that of an untreated control. Furthermore, NAC prevented the functional changes to acrolein or CAA in the cells, with levels of all mediators being the same as untreated controls. These are promising results as it appears NAC can fully protect urothelial cells from acrolein or CAA damage.

Few studies have looked at the ability of NAC to protect urothelial cells from CPO or IFO induced toxicity. In exfoliated bladder cells from mice treated with CPO, with and without oral NAC supplementation, it was shown that genotoxicity, inflammatory infiltration and haemorrhage were prevented by NAC (Gurbuz et al., 2009). However, NAC has shown similar protective effects in other tissues. For example, NAC has been shown to protect rats treated with IFO from nephrotoxicity, improving GSH levels and preventing morphological damage to renal tubules and glomeruli (Chen et al., 2008) without affecting its antitumor activity (Hanly et al., 2012). NAC (400 μ M) also protected porcine renal epithelial cells from IFO induced toxicity. (Chen et al., 2007). Rat hepatocytes were protected from acrolein (100 μ M) and CAA (300 μ M) induced toxicity and ROS production by NAC (1mM) (Macallister et al., 2012) while acrolein toxicity in primary human hepatocytes was also attenuated by NAC (Mohammad et al., 2012)

Tanel and Averill-Bates (2007) demonstrated that the mechanism by which NAC protected chinese hamster ovary cells from acrolein cytotoxicity was by increasing intracellular GSH and preventing apoptosis. Furthermore, NAC has been shown to stop ROS induced apoptosis in human bladder urothelial cells due to sulforaphane and compound K (Jo et al., 2014, Wang et al., 2013b). As described in the results above, NAC was able to prevent the increase in caspase-3 activity due to acrolein and CAA treatment of human urothelial cells supporting an anti-apoptotic mechanism of action. However, given that NAC also reduced ROS production in both cells lines also suggests that the mechanism of NAC protection may be preventing

oxidative stress. From these results, NAC appears to be a promising alternative or addition to current uroprotective co-treatments for CPO and IFO therapy. NAC not only appears to protect urothelial cells from damage but also from the functional changes induced by acrolein and CAA.

NAC given orally is excreted in the urine in the form of both NAC and cysteine (Ventura et al., 2003) making it possible that a simple oral dose of NAC could protect patients from acrolein and CAA induced urotoxicity. For patients this could mean oral supplementation during and after treatment with CPO or IFO could reduce the pain, urinary frequency and urgency associated with these drugs and potentially improve treatment tolerability and outcomes.

Conclusions

This is the first study to report the effect of acrolein and CAA in human urothelial cells. Both acrolein and CAA are toxic to human urothelial cells and induce increased ROS production, apoptosis and changes in mediator release. This study supports a role for CAA in the urotoxicity of CPO and IFO as it was not only damaging to urothelial cells but more toxic than acrolein. Furthermore, this study has shown that NAC, but not vitamin C, appears to protect urothelial cells from the acrolein and CAA induced changes and accordingly may be useful in preventing urotoxicity in patients receiving CPO and IFO chemotherapy.

CHAPTER 4: Effects of Acrolein or Chloroacetaldehyde on the Function of Isolated Porcine Bladder

4.1 INTRODUCTION

The previous Chapter has demonstrated that the CPO and IFO metabolites acrolein and CAA are capable of damaging human urothelial cells and changing their release of ATP, ACh, PGE₂ and cytokines. However, it raises the question as to whether these metabolites cause the same changes in normal bladder tissue with intact urothelial, lamina propria and detrusor layers and if so, do they alter the function of the urothelium or the detrusor? This study aimed to address these questions and further investigate the effects of acrolein and CAA on bladder function using isolated porcine tissues.

Comparison of Human and Pig Bladder

The properties of the pig lower urinary tract, including efferent control, urodynamic, histochemical and immunohistochemical characteristics, have been shown to be similar to human bladder in many respects (Bridgewater et al., 1993, Crowe and Burnstock, 1989, Sibley, 1984). In humans, bladder contraction is mediated almost completely by ACh from parasympathetic nerves acting via the muscarinic (M) receptors. In mouse and human bladder the M2 receptor density is three times greater than that of the M3 receptor (Wang et al., 1995), however direct bladder contraction is almost entirely mediated by the M3 receptor (Chess-Williams, 2002, Fetscher et al., 2002). Yamanishi et al. (2000) found the muscarinic receptor expression in pig bladder to be similar to that of humans with the M2 subtype predominating (70-80%) over the M3 subtype, while contraction of the pig bladder was mediated by the M3 receptor (Sellers et al., 2000, Yamanishi et al., 2000).

Both human and pig bladder can also contract in response to ATP and the smooth muscle cells of both species develop similar inward currents in response to purinoceptor activation (Inoue and Brading, 1991, Tsai et al., 2007). Although ATP is co-released with ACh from parasympathetic nerves in humans, it appears to have very little contribution to nerve mediated contraction. However, in pig bladder atropine inhibits the contractile response by approximately 80% with a small NANC contraction remaining, thought to be due to ATP (Sibley, 1984, Inoue and Brading, 1991). The P2X1 is the most abundant P2X receptor subtype in the human detrusor (O'Reilly et al., 2001) as well as the pig (Bahadory et al., 2013).

Noradrenaline from the sympathetic nerves has an inhibitory action on detrusor allowing the muscle to remain relaxed during filling. Human detrusor can express all three β -AR subtypes (Andersson and Arner, 2004), however it is the β 3-AR which is mainly responsible for the relaxation response (Igawa et al., 1999, Takeda et al., 1999, Igawa et al., 2001, Yamaguchi,

2002, Badawi et al., 2007). Similarly, β 3-ARs have also been demonstrated in the detrusor of the pig and are the predominant mediators of relaxation to isoprenaline (Yamanishi et al., 2002c, Yamanishi et al., 2002d).

Detrusor smooth muscle of pig has been shown to have similar electrical properties to those of humans, both exhibiting nifedipine (10 μ M) sensitive spontaneous action potentials followed by fast hyperpolarisations that can be blocked by apamin (0.1 μ M) (Hashitani and Brading, 2003). Furthermore, spontaneous contractile activity is similar in both human and pig tissue (Sibley, 1984).

Several studies of urothelial structure and function have demonstrated that human and porcine tissues share many similar features (Janssen et al., 2013, Kumar et al., 2004). The urothelium forms a barrier protecting the bladder wall but also plays a role in bladder function. Studies of mammalian uroplakin expression have shown that pig and human (among other mammals) both express the four uroplakins (Ia, Ib, II and III) and the urothelial plaques containing them appear morphologically similar (Wu et al., 1994). Furthermore, immunofluorescence studies in human and porcine urothelium demonstrated that the GAG expression and distribution are comparable between species (Janssen et al., 2013).

Mechanical stretch during bladder filling stimulates the urothelium to release mediators which can communicate with underlying cells such as sensory nerves, interstitial cells and smooth muscle (Birder et al., 2010b) and disruption of these mechanisms is associated with bladder dysfunction (Kumar et al., 2010, Chen et al., 2003, Sun and Chai, 2006, Sun et al., 2001). Functionally, the release of ATP from the urothelium of human and porcine bladders in response to mechanical stretch and electrical stimulation is comparable (Kumar et al., 2004). The pig bladder is structurally and functionally similar to that of the human and provides a good model for studying the effects of CPO and IFO on the human bladder.

The Effects of CPO and IFO on the Bladder

The urotoxic effects of CPO or IFO include anatomical and functional effects. The metabolites of CPO and IFO come into direct contact with the urothelium causing damage and resulting in inflammation which presents as bladder oedema, ulceration, neovascularisation and haemorrhage (Cox, 1979, Lima et al., 2007). The functional effects can manifest as bladder pain, urinary frequency, urgency and feelings of incomplete emptying (Korkmaz et al., 2007, Fukuoka et al., 1991) which can persist well beyond the resolution of inflammation and damage.

Most of the studies into the effects of CPO and IFO on the bladder have been performed in rodents and more work is needed in humans or large mammals such as the pig. Isolated bladder tissue from rats treated with CPO or IFO demonstrated reduced contraction in response to KCl and carbachol suggestive of damage to bladder smooth muscle (Macedo et al., 2011, Giglio et al., 2005). However, the muscarinic receptor expression of the muscle was altered after treatment suggesting that functional changes may involve more than just reduced muscle contractility (Giglio et al., 2005). Similarly, contractile responses to the P2X receptor agonist β,γ -methylene ATP (β,γ -mATP) were reduced in CPO treated animals and may also be due to changes in receptor expression (Mok et al., 2000, Kageyama et al., 2008). However, the question remains as to whether acrolein, CAA or both these metabolites contribute to these changes. Furthermore, it is the efferent nerves that control the bladder in physiological conditions and changes to their function has yet to be investigated.

Changes in mediator release from the urothelium have been reported in various painful or overactive bladder conditions. Previous studies in rodents have demonstrated that systemic CPO treatment resulted in increased urothelial ATP release in response to hypo-osmotic stimulation (Smith et al., 2005). Furthermore, COX-2 expression in the urothelium and plasma PGE₂ concentration in rats were increased 12-24 hours after administration of IFO (Macedo et al., 2011). Again, it is unclear whether these changes occur in humans and whether acrolein or CAA is responsible.

Evaluation of Bladder Function

Isolated strips of pig bladder have been extensively used as a model to study human bladder function (Moro et al., 2011, Sadananda et al., 2008, Kumar et al., 2004, Templeman et al., 2003, Yamanishi et al., 2002d, Yamanishi et al., 2000, Sibley, 1984). Recently, a technique of mounting full thickness bladder sections in a bath to allow separate solutions to bath the luminal and serosal surfaces has been described (Smith et al., 2014). This technique allows the urothelial surface to be treated relatively selectively, imitating the physiological condition of CPO and IFO toxicity where the toxic metabolites are concentrated in the urine on the luminal surface. After treatment the bladder sections can be dissected into strips and used in conventional functional studies.

Aims

The aim of the present study was to investigate the effect of acrolein or CAA treatment on the porcine urothelium and detrusor. Specific aims were:

- To examine the effect of acrolein or CAA on urothelial structure;
- To determine the effect of acrolein or CAA on urothelial mediator release;
- To investigate whether acrolein or CAA treatment changes the contractility of the urothelium or detrusor;
- To determine whether acrolein or CAA treatment causes alterations in the efferent nerve responses in the detrusor.

4.2 METHODS

Animals

Mature adult female pig (sow) bladder samples were obtained from a local abattoir. The tissues were immediately placed in Krebs-bicarbonate solution (composition in mmol/L: NaCl 118.4, NaHCO_3 24.9, KCl 4.7, CaCl_2 1.9, MgSO_4 1.15, KH_2PO_4 1.15, glucose 11.7) at 4°C until use.

Experimental Setup and Procedures

The bladder was opened longitudinally from the urethra to the dome and sections cut from the dome of the bladder and mounted in modified Ussing chambers (as previously described by Smith et al., (2014)) containing Krebs bicarbonate solution at 37°C and gassed with 5% CO_2 in oxygen (**Figure 4.1**). Krebs bicarbonate solution containing acrolein (100 μM) or chloroacetaldehyde (10 μM) was applied to the urothelial side of the tissue. A volume of 5mL was applied to fully cover the urothelial surface and was refreshed after 2 hours. A control tissue was also treated simultaneously with normal Krebs bicarbonate solution. Tissues were unable to be treated for 24 hours due to loss of function after this time, therefore a shorter treatment period of 4 hours was used.

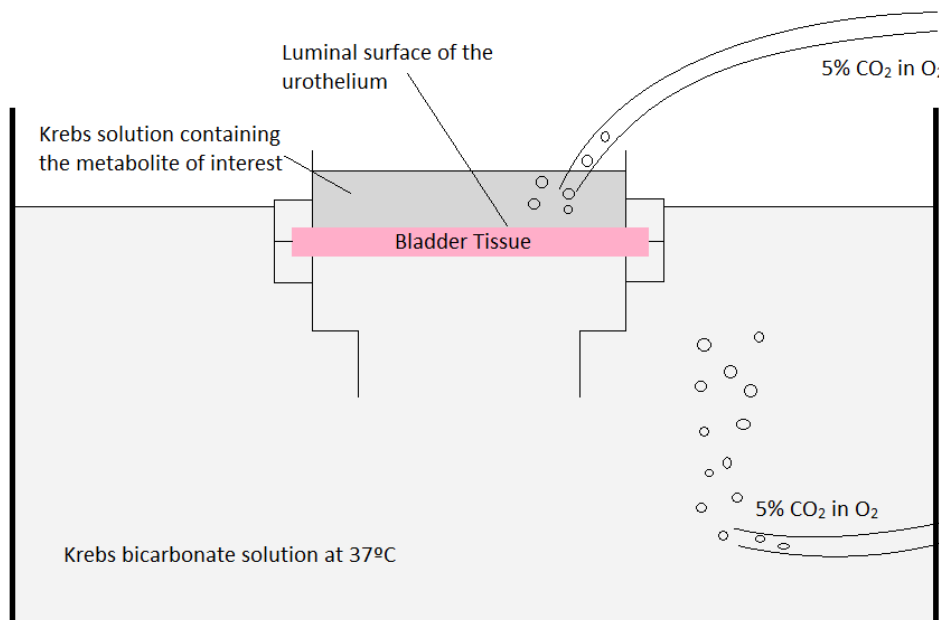


Figure 4.1: Schematic figure of the modified Ussing chamber with a piece of bladder tissue secured within the chamber in Krebs solution at 37°C and gassed with 5% CO_2 in oxygen. The luminal surface of the urothelium is treated with a Krebs solution alone or containing acrolein (100 μM) or chloroacetaldehyde (10 μM).

After treatment, the tissues were washed with Krebs solution and the bladder dome dissected into intact and denuded detrusor strips (with and without urothelium/lamina propria) and isolated urothelial/LP strips. Orientation of the tissues was marked so that tissue strips were cut longitudinally from the tissue approximately 10mm long and 5mm wide.

The treated and control tissue strips were anchored in separate organ baths at 37°C and gassed with 5% CO₂ in oxygen. Tissues were equilibrated for 1 hour during which time they were washed with fresh Krebs-bicarbonate solution every 15 minutes (**Figure 4.2**). Tissue strips were connected to an isometric force transducer (ADInstruments, Ltd Australia) under 20mN resting tension. The isometric force transducer was connected to a Powerlab 8/30 recording system (ADInstruments Ltd.) via an Octal Bridge Amp (ADInstruments Ltd) and the developed tension was measured using LabChart (version 7.0.3) software (ADInstruments Ltd).

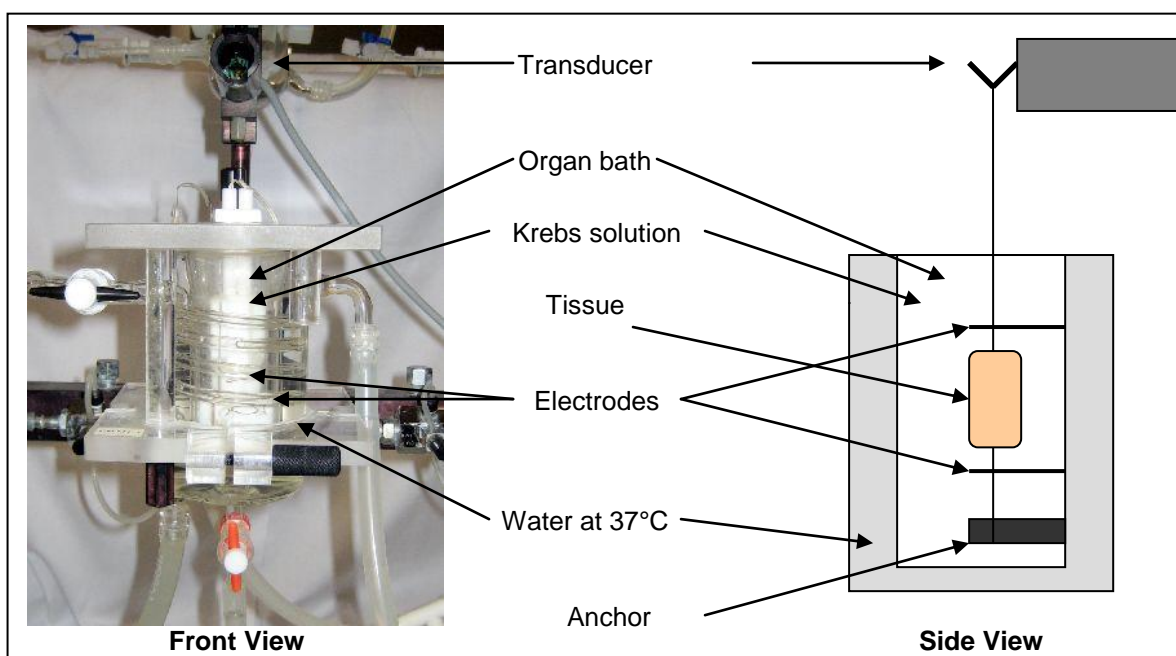


Figure 4.2: An organ bath with an isometric force transducer positioned above to record tension developed by the tissue. The side view is an illustration of the manner in which the tissue strip is set up in the organ bath, anchored at the base between two electrodes and attached to the transducer.

Response to Pharmacological Agents

After equilibration, tissues were exposed to various agonists and responses recorded as changes in tension from baseline, baseline being taken as the maximum tension recorded in the 60 seconds prior to administration of the drug. The agonists investigated included single concentrations of potassium chloride (KCl) (60mM) and ATP (1mM), and a cumulative concentration-response curve to carbachol (6nM – 180µM). Next, a submaximal dose of carbachol was applied (36.5µM) and after the contraction had reached a plateau a cumulative concentration-response curve to isoprenaline (45pM - 45µM) was performed. Responses from

treated tissues were compared to control responses to determine if acrolein or chloroacetaldehyde affected the contractility of the detrusor or the urothelium/LP. Responses from the detrusor strips with and without the urothelium/LP were compared to test the inhibitory influence of the urothelium/LP.

Spontaneous Contractions

As discussed above, the detrusor and the urothelium/LP develop spontaneous, phasic contractions and these were observed in the isolated porcine bladder strips (**Figure 4.3**). The frequency and amplitude of spontaneous contractions were quantified. Spontaneous contractions were measured during 200 seconds at the end of the initial equilibration period. Average frequency was represented as contractions/min and average amplitude in mN.

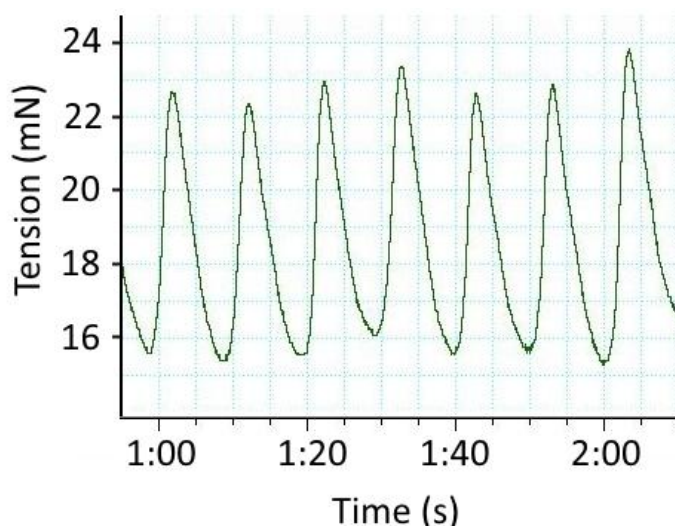


Figure 4.3: Typical trace showing spontaneous contractions of a strip of isolated porcine urothelium/lamina propria during 60s.

Urothelial Transmitter Release

Urothelium/lamina propria tissue strips were prepared and set up in organ baths as discussed above. Following equilibration, 2mL of fresh Krebs was added to the bath and collected after 2 minutes for a basal sample of mediator levels. The Krebs was immediately refreshed and the tissue stretched to 50% of its original length over 2 minutes at which time the Krebs was collected for measurement of stimulated mediator release. The level of ATP, ACh and PGE₂ in each sample was measured using the assays described in Chapter 2.

Electrical Field Stimulation

Denuded detrusor strips were prepared and set up as discussed above. The tissue strips were electrically stimulated (40 volts and 1ms pulse-width) delivered as a 5 second train every 100 seconds, at a frequency of 1Hz, 5Hz, 10Hz and 20Hz. The tissues were stimulated at each

frequency until stable contractions were obtained. Next, tissues were stimulated at each frequency in the presence of 100 μ M L-NNA (NOS inhibitor), 1 μ M atropine (muscarinic antagonist) and 10 μ M $\alpha\beta$ mATP (desensitises purinoceptors) to investigate the contribution of NO, ACh and ATP to the responses. Responses in treated tissues were compared to controls.

Histology

After treatment, tissues were dissected and placed in neutral buffered formalin (10%), processed and stained as per the protocol in Chapter 2 in order to visualise any tissue damage caused by acrolein or CAA.

Statistical Analysis

Results were expressed as mean \pm standard error of the mean (SEM). Data were analysed using a Student t-test or one-way ANOVA with Dunnett or Tukey multiple comparisons test as appropriate, using Graphpad InStat (version 3.06) software (SanDiego, CA). Comparisons of whole response curves of control and treated groups were performed using two-way ANOVA with Bonferroni post-test. Significance levels were defined as $p < 0.05$ (*), $p < 0.01$ (**) and $p < 0.001$ (**).

4.3 RESULTS

Effect of Acrolein and CAA on Urothelium/Lamina Propria Structure and Mediator Release

The effect of acrolein and CAA on porcine bladder was first investigated in terms of their damaging effects on the structure of the urothelium. Representative haematoxylin and eosin stained sections of control, acrolein (100 μ M) and CAA (10 μ M) pre-treated bladders are shown (**Figure 4.4**). Typical histological features were identifiable in sections of control tissues with the folded urothelium and lamina propria layers overlying a deeper smooth muscle layer. However, minor damage was evident in acrolein and CAA treated tissues. Both metabolites appeared to cause some thinning of the urothelial layer but this was not significant (**Figure 4.5**). In addition, CAA (10 μ M) treatment appeared to cause some disruption to the normal folding of the urothelial layer while the folding was absent in acrolein (100 μ M) treated bladders.

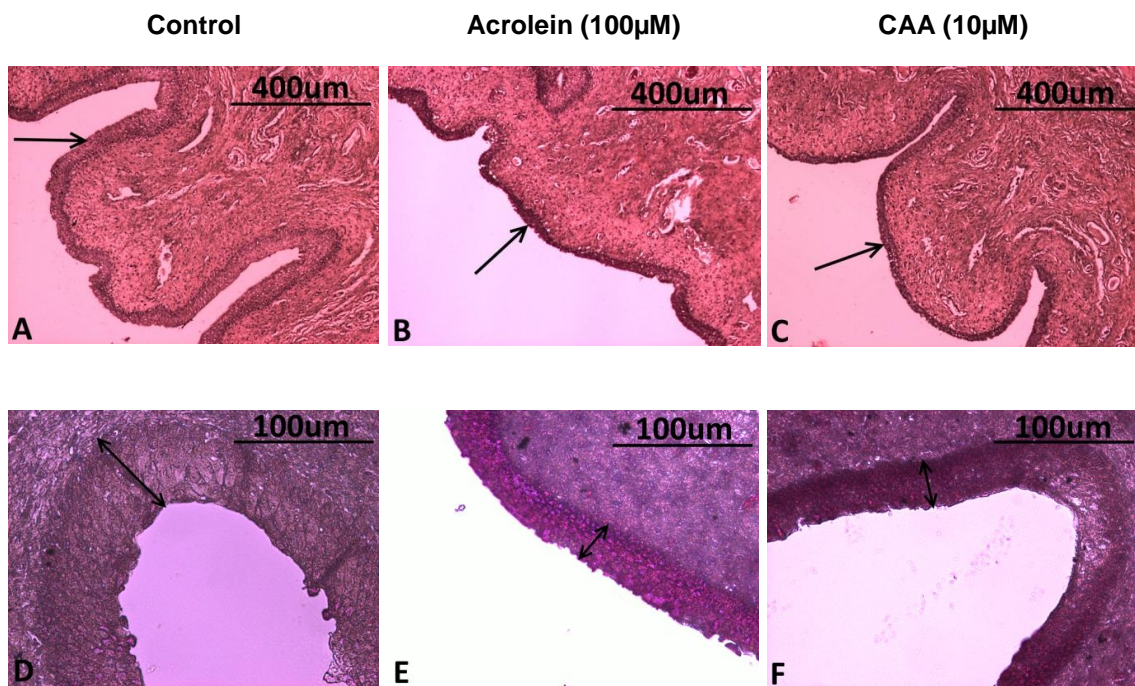


Figure 4.4: Histological sections of isolated porcine bladder from control tissue (A & D) and tissue treated with acrolein (100 μ M) (B & E) and CAA (10 μ M) (C & F) at 10x and 40x magnification respectively. The urothelium is indicated by an arrow.

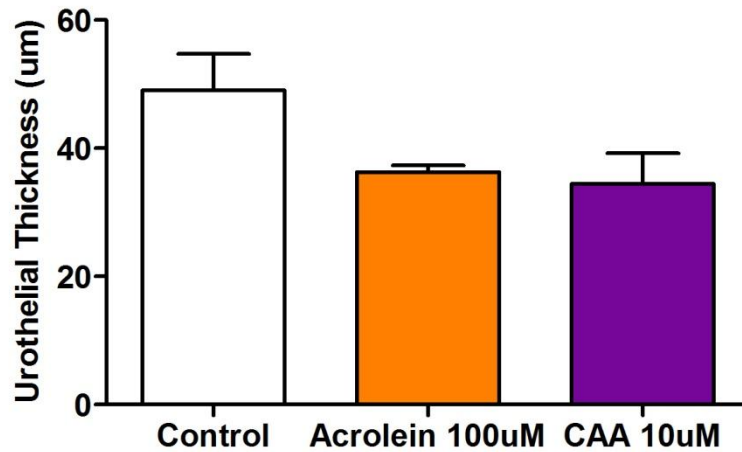


Figure 4.5: Urothelial thickness (um) in control tissue (A) and tissue treated with acrolein (100μM) (B) and CAA (10μM) (C). Data represented as mean ± SEM (n=3).

Next, the release of mediators from the urothelium/LP after acrolein and CAA treatment was investigated. Initially the repeatability of mediator release was determined by performing the basal and stretch sample collection three times with a 15 minute recovery between each repetition. Samples were tested for ATP and ACh content. As shown in **Figure 4.6**, ATP and ACh levels in stretch samples were not significantly different from levels in basal samples. Pre-incubation with luminal acrolein (100μM) or CAA (10μM) for four hours had no significant effect on the release of ATP, ACh or PGE₂ during basal or stretch conditions (**Figure 4.7**).

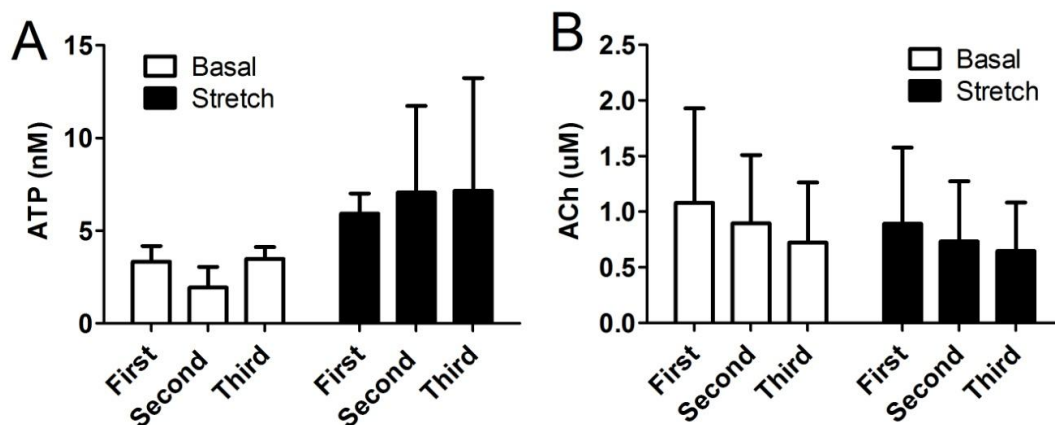


Figure 4.6: Repeatability of urothelial ATP (A) and ACh (B) release from isolated porcine tissue during three replicates of basal and stretch conditions.

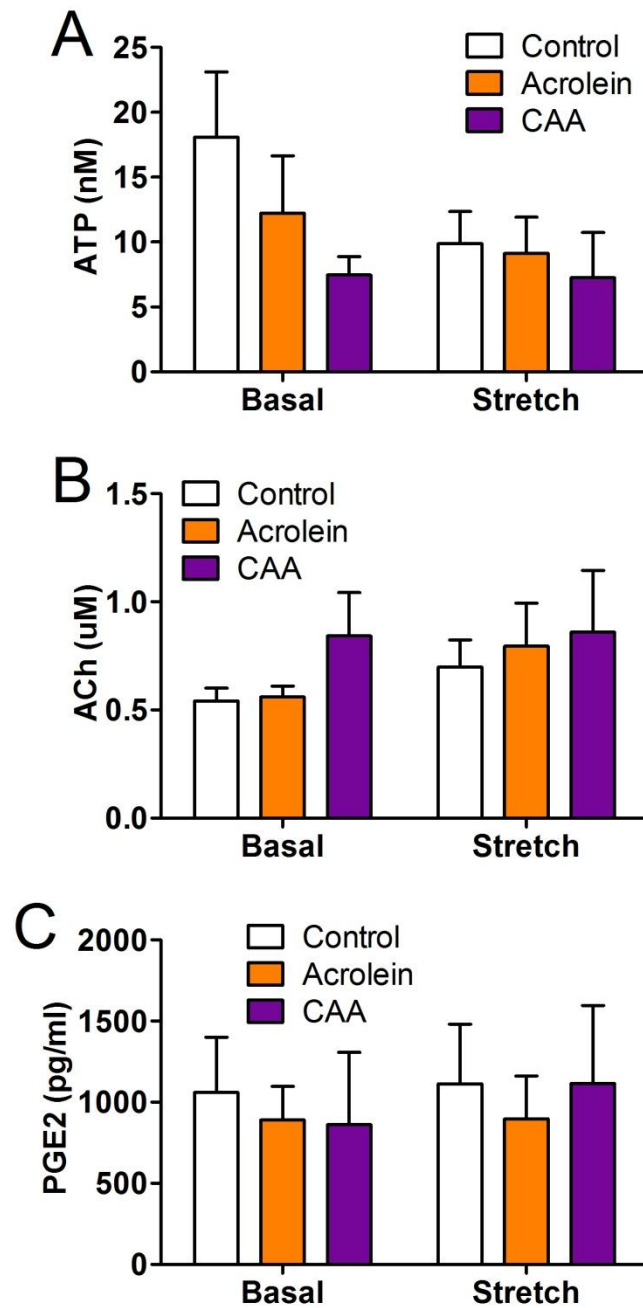


Figure 4.7: Basal and stretch induced ATP (A) acetylcholine (B) and PGE₂ (C) levels in isolated strips of urothelium/lamina propria from bladder previously incubated with luminal acrolein (100μM), CAA (10μM) or krebs for 4 hours. Data are shown as mean ± SEM (n=6).

Effect of Acrolein or CAA Treatment on the Contractility of Tissue Strips

The effect of acrolein and CAA on isolated porcine bladder tissue contractility was then investigated using urothelium/lamina propria, denuded and intact detrusor strips. Firstly, the maximum response to KCl (60mM), ATP (1mM) and carbachol (180 μ M) were determined followed by the tissue response to full dose response curves to carbachol (6nM – 180 μ M) and isoprenaline (45pM - 45 μ M).

The contractility of the urothelial/LP strips were unaffected by treatment with acrolein or CAA. Responses of detrusor strips to carbachol, KCl and ATP were all lower in tissues from acrolein or CAA treated bladders than control incubated bladders although none of the differences reached statistical significance (**Figure 4.8**). Specifically, the detrusor response to KCl was decreased by approximately 30%, the carbachol response by 15% and the ATP response by approximately 50% after treatment with either metabolite. However, the decreased contractility after acrolein or CAA treatment was not observed in the presence of the urothelium/lamina propria with similar responses observed in treated and controls strips.

The presence of the urothelium/LP significantly inhibited the contractile responses of the control detrusor to KCl and ACh ($p < 0.05$). Responses to ATP also tended to be lower in urothelium intact tissues but this was not statistically significant. Pre-treatment with acrolein or CAA did not change the inhibitory effect of the urothelium/LP on detrusor responses to KCl. Specifically, the presence of the urothelium/LP inhibited the contraction of the control detrusor strips to KCl by $49 \pm 8\%$ ($p < 0.05$) and acrolein or CAA treated contractions were inhibited by $48 \pm 19\%$ and $50 \pm 10\%$ ($p < 0.05$) respectively.

However, after pre-treatment with acrolein or CAA there was no statistically significant difference between detrusor responses to ACh or ATP in the presence or absence of the urothelium/lamina propria. In the presence of the urothelium/LP detrusor responses to carbachol were significantly inhibited in controls strips [by $44 \pm 5\%$ ($p < 0.05$)] whereas no significant effect was observed in acrolein or CAA treated strips (inhibited by $31 \pm 11\%$ and $28 \pm 12\%$ respectively). The control tissue responses to ATP were inhibited by $52 \pm 14\%$ ($p < 0.05$) in the presence of the urothelium/LP whereas no inhibitory effect was observed in acrolein or CAA treated tissues.

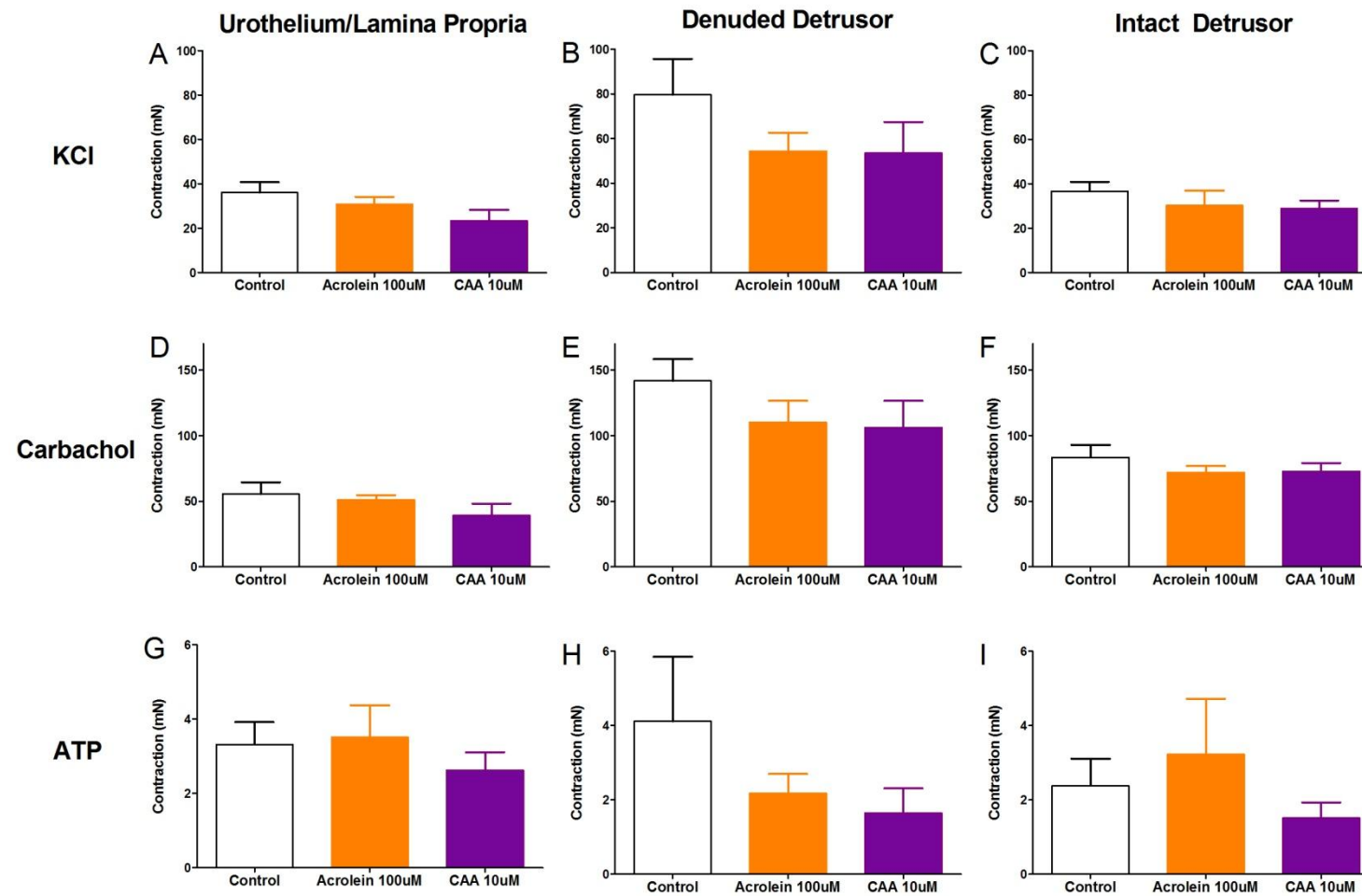


Figure 4.8: Effect of acrolein (100uM) and chloroacetaldehyde (CAA) (10uM) pre-treatment on the contractility of porcine urothelium/lamina propria and denuded and intact detrusor in response to KCl (60mM) (A, B & C respectively), carbachol (180uM) (D, E & F respectively) and ATP (1mM) (G, H & I respectively). Data represented as mean \pm SEM (n=6) and analysed by one-way ANOVA with Dunnett post-test.

Next concentration-response curves to carbachol and isoprenaline were performed. As the concentration of carbachol increased so did the contraction of the urothelial/lamina propria, denuded and intact detrusor strips (**Figure 4.9**). The contractility of the urothelial/LP and intact detrusor strips to cumulative concentrations of carbachol was not different after acrolein (100 μ M) or CAA (10 μ M) pre-treatment (**Figure 4.10 A-C**). However, the treated denuded detrusor demonstrated slightly less contraction over the entire curve but this was not significant. Again, the presence of the urothelium inhibited the control detrusor contractions to carbachol ($p < 0.05$) but did not significantly affect detrusor contractions in tissue treated with acrolein or CAA (**Figure 4.11**). The pEC_{50} values of carbachol in each tissue type and treatment group were also similar (**Figure 4.12 & Table 4.1**).

Tissue tension decreased as the isoprenaline concentration increased (**Figure 4.10 D-F**). The response of the urothelial/LP and intact detrusor strips were unchanged by acrolein (100 μ M) or CAA (10 μ M) treatment. However, the denuded detrusor again appeared less responsive after treatment but the difference was not statistically significant. Acrolein (100 μ M) or CAA (10 μ M) treatment had no effect on the pEC_{50} of isoprenaline in the various tissues (**Figure 4.12 & Table 4.1**).

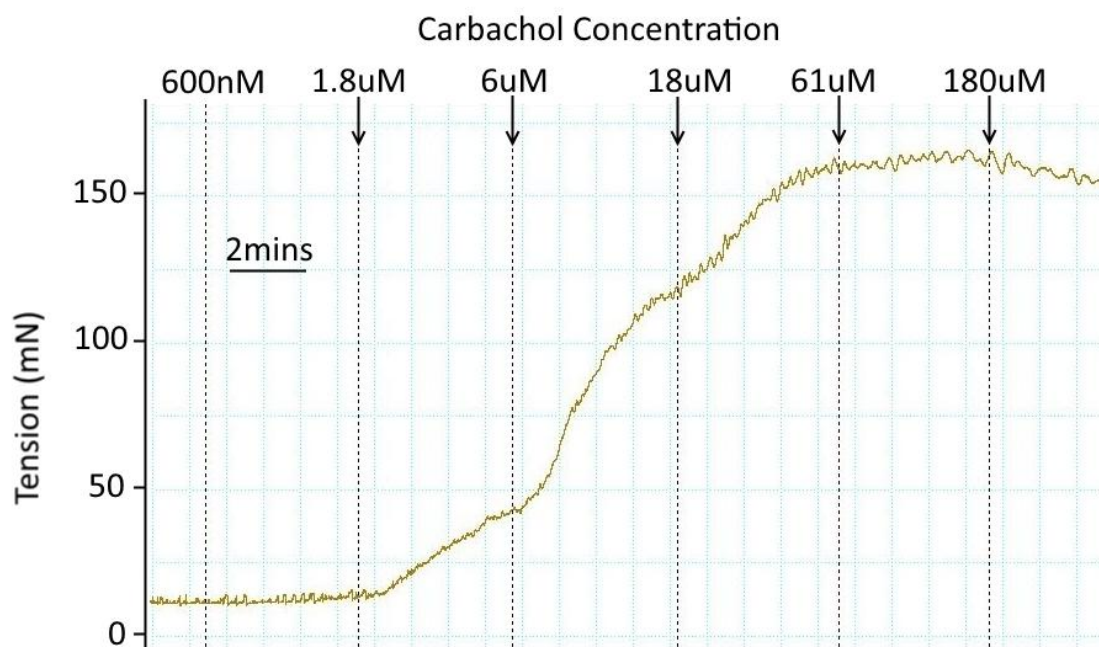


Figure 4.9: Representative trace of isolated porcine detrusor to cumulative concentrations of carbachol.

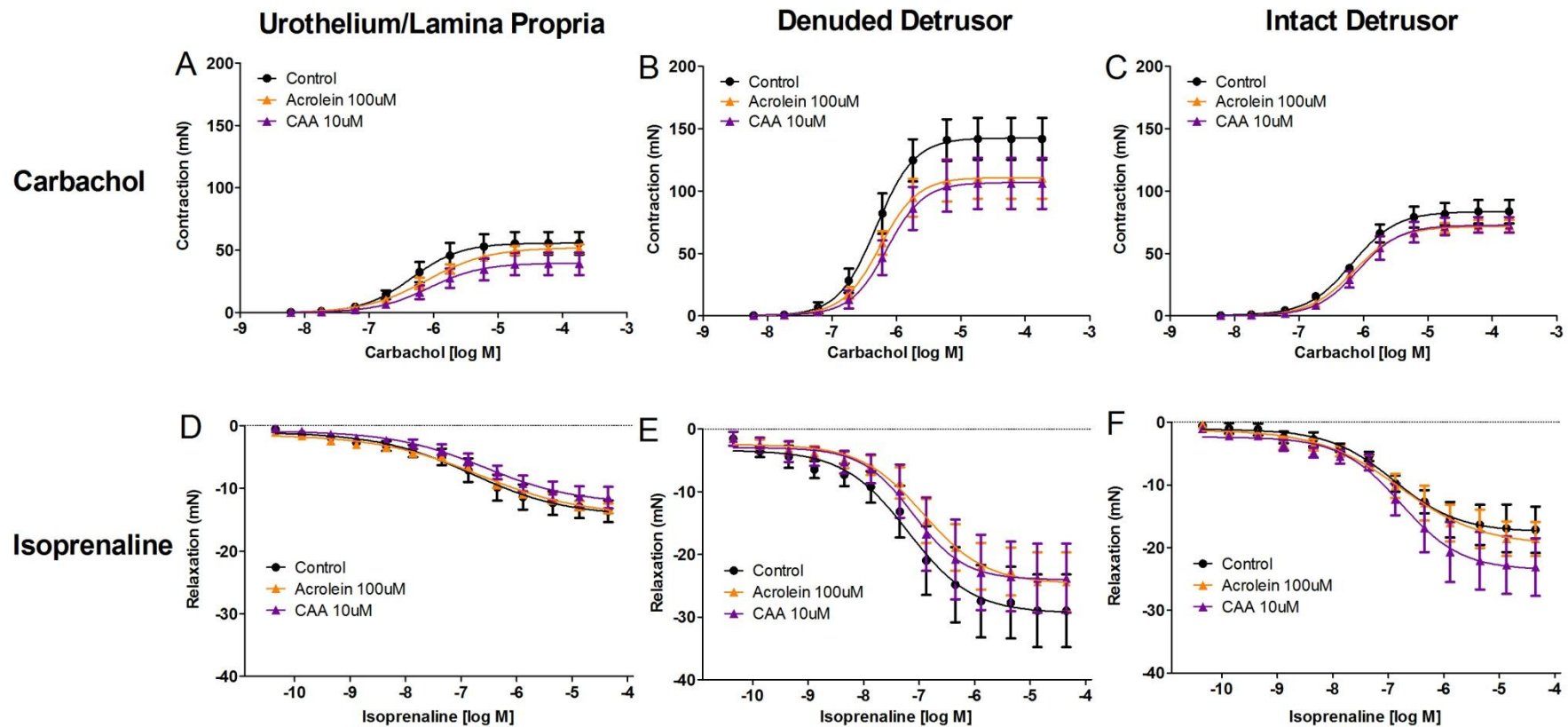


Figure 4.10: Cumulative concentration response (mN) curves to carbachol (A, B & C) and isoprenaline (D, E and F) in strips of porcine urothelium/lamina propria, denuded detrusor and intact detrusor pre-incubated with Krebs (control), acrolein (100 μ M) or CAA (10 μ M) for four hours. Data represented as mean \pm SEM (n=6).

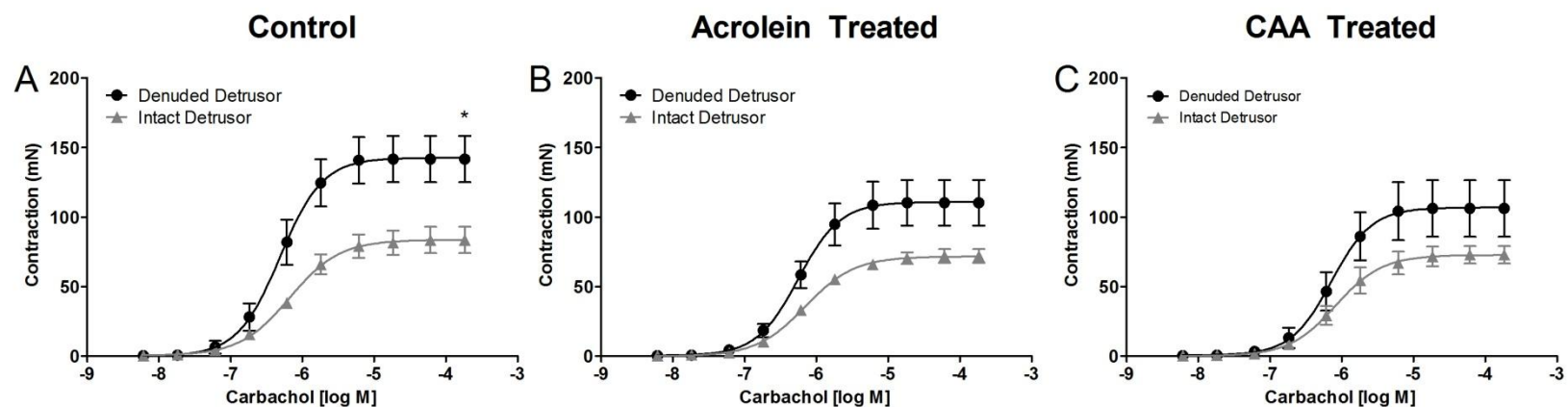


Figure 4.11: The inhibitory effect of the urothelium/lamina propria on detrusor contractions in response to cumulative concentrations of carbachol in strips of porcine detrusor pre-incubated with Krebs (control) (A), acrolein (100 μ M) (B) or CAA (10 μ M) (C) for four hours. Data represented as mean \pm SEM (n=6). Maximum response of denuded and intact detrusor within each treatment group were compared by paired two-tailed t-test (* indicates $p < 0.05$ vs intact response).

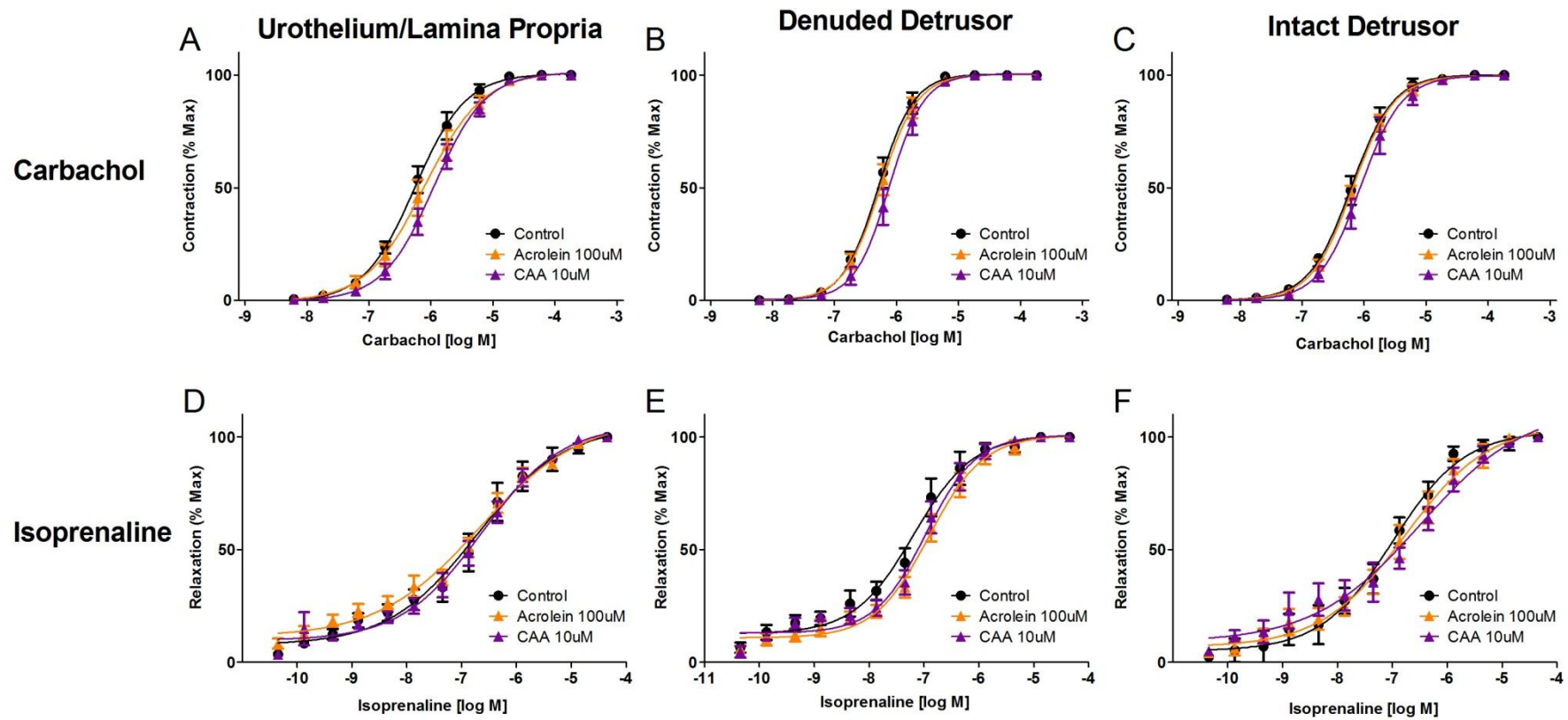


Figure 4.12: Percentage of maximum response to cumulative concentrations of carbachol (A, B & C) and isoprenaline (D, E and F) in strips of porcine urothelium/lamina propria, denuded detrusor and intact detrusor pre-incubated with Krebs (control), acrolein (100 μ M) or CAA (10 μ M) for four hours. Data represented as mean \pm SEM (n=6).

Table 4.1: The pEC50 values for carbachol and isoprenaline in porcine urothelium/lamina propria, denuded detrusor and intact detrusor.			
	Urothelium/LP	Denuded Detrusor	Intact Detrusor
<i>Carbachol</i>			
Control	6.26±0.05	6.30±0.03	6.21±0.04
Acrolein (100µM)	6.11±0.07	6.26±0.03	6.18±0.02
CAA (10µM)	5.96±0.04	6.12±0.04	6.07±0.05
<i>Isoprenaline</i>			
Control	6.73±0.15	7.19±0.09	6.99±0.12
Acrolein (100µM)	6.67±0.13	6.91±0.07	6.77±0.13
CAA (10µM)	6.63±0.11	6.97±0.07	6.49±0.24
Data represented as mean ± SEM (n=6)			

Effect of Acrolein or CAA Treatment on the Spontaneous Activity of Tissue Strips

Spontaneous contractions of the detrusor and urothelium/LP after one hour equilibration of tissue strips were measured. Both frequency and amplitude of the spontaneous contractions were significantly higher in urothelial/LP tissues than in detrusor tissues (**Figure 4.13**). Treatment with acrolein (100 μ M) or CAA (10 μ M) had no significant effect on the average frequency (**Figure 4.13-A**) or the amplitude (**Figure 4.13-B**) of spontaneous contractions compared to control.

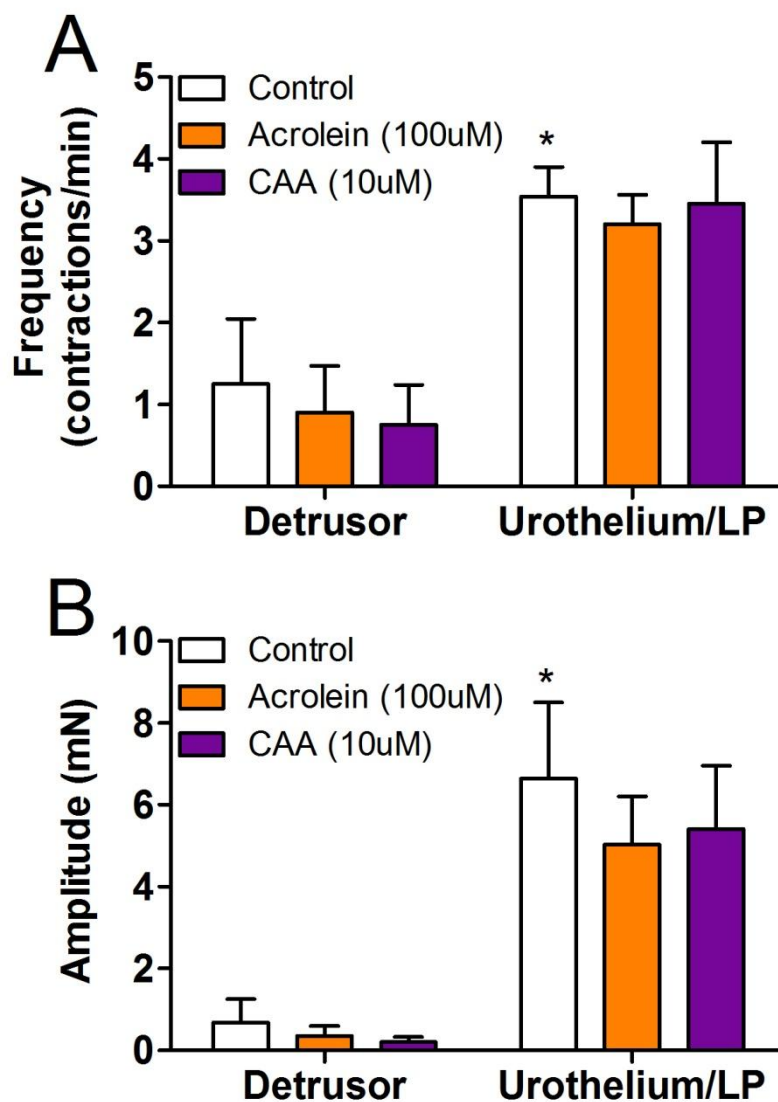


Figure 4.13 : Frequency and amplitude of spontaneous contractions of porcine detrusor and urothelium/lamina propria from control, acrolein (100 μ M) and CAA (10 μ M) pre-treated tissues. Data represented as mean \pm SEM (n=6). Control detrusor and urothelial responses were compared by unpaired two-tailed t-test (*p<0.05).

Effect of Acrolein or CAA Treatment on Responses to Electrical Field

Stimulation

Electrical field stimulation was used to investigate the effect of acrolein or CAA treatment on efferent nerve function in the porcine bladder. As the frequency of stimulation increased, the contractile response of the detrusor also increased (**Figure 4.14-A**). The EFS induced contractions were sensitive to tetrodotoxin (TTx) ($1\mu\text{M}$) with responses being completely abolished in the presence of this neurotoxin (**Figure 4.14-B**). Removing NO with L-NNA ($100\mu\text{M}$) had no effect on the nerve mediated response (**Figure 4.15**). Antagonism of the muscarinic receptors with atropine ($1\mu\text{M}$) almost completely abolished the EFS induced response leaving a small contraction of less than 10% of the control response at 20Hz (**Table 4.2**). Desensitisation of the purinergic receptors with $\alpha\beta\text{mATP}$ ($10\mu\text{M}$) had no significant effect on the remaining contraction (**Figure 4.15**). However, as indicated in **Table 4.2**, $\alpha\beta\text{mATP}$ tended to partially block contraction responses at low frequencies (1Hz) but did not affect responses at high frequencies (10-20Hz).

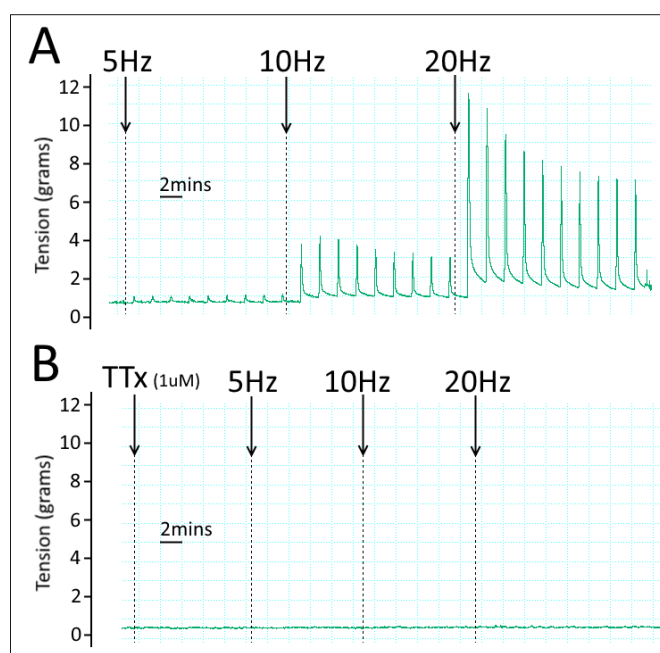


Figure 4.14: Typical trace of untreated porcine bladder responses to electrical field stimulation (40 volts, 1ms pulse-width, 5 second train) in the absence (A) and presence (B) of tetrodotoxin (TTx) ($1\mu\text{M}$).

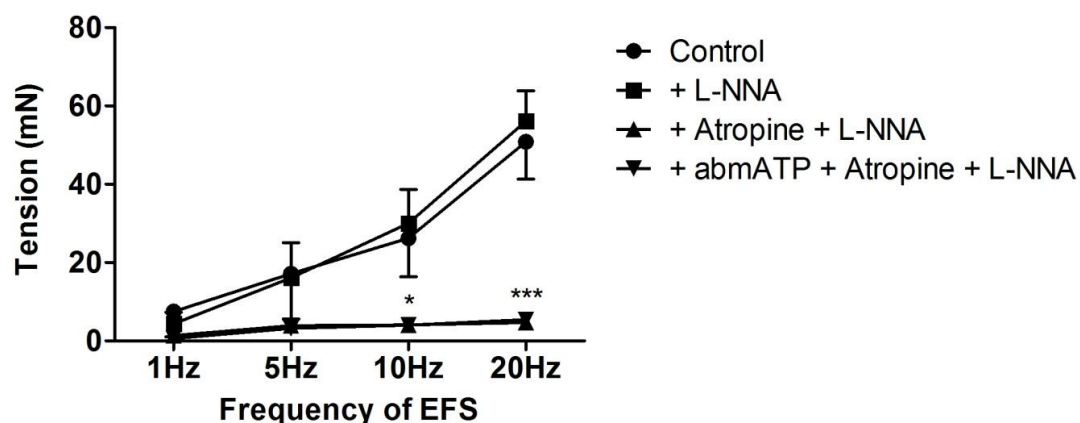


Figure 4.15: Contractile responses of untreated porcine detrusor strips to electrical field stimulation (40 volts, 1ms pulse-width, 5 second train). Data represented as mean \pm SEM (n=6). Analysed by one-way ANOVA with Dunnetts post-test (* p <0.05, *** p <0.001 atropine + LNNA vs control).

Table 4.2: Response (mN) to EFS in the absence and presence of L-NNA (100 μ M), Atropine (1 μ M) and α β mATP (10 μ M).				
	1Hz	5Hz	10Hz	20Hz
Control	7.5 \pm 6.5	17.2 \pm 11.5	26.2 \pm 9.9	50.8 \pm 9.5
+ L-NNA (100 μ M)	4.4 \pm 3.0	16.1 \pm 9.0	30.0 \pm 8.7	56.1 \pm 7.8
+ Atropine (1 μ M) + L-NNA (100 μ M)	1.4 \pm 0.9	3.9 \pm 1.6	4.1 \pm 1.6	4.7 \pm 1.9
+ α β mATP (10 μ M) + Atropine (1 μ M) + L-NNA (100 μ M)	0.7 \pm 0.4	3.2 \pm 1.2	4.0 \pm 1.5	5.4 \pm 1.9
Data represented as mean \pm SEM (n=6).				

Treatment with acrolein (100 μ M) or CAA (10 μ M) had no significant effect on the response to EFS (Figure 4.16). No change in the contribution of NO, ACh, or ATP was seen after treatment with acrolein (100 μ M) or CAA (10 μ M). ACh was the major contributor to the EFS induced contraction at all frequencies in all treatment groups however the influence of ACh tended to increase as the frequency increased (approximately 70% at 5Hz, 85% at 10Hz and 90% at 20Hz) (Figure 4.17).

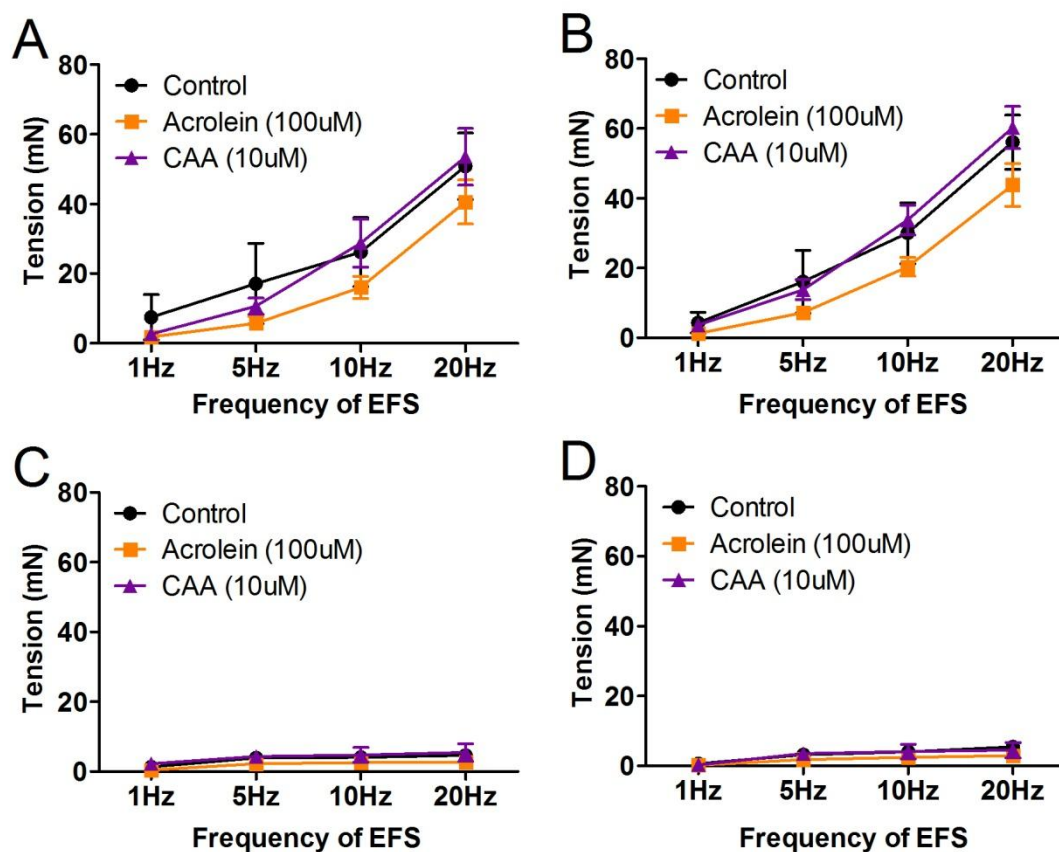


Figure 4.16: Contractile responses of porcine detrusor strips treated with Krebs (control), acrolein (100 μ M) or CAA (10 μ M) to electrical field stimulation (40 volts, 1ms pulse-width, 5 second train) in the absence of antagonists (A), in the presence of L-NNA (100 μ M) (B) and atropine (1 μ M) (C) and also $\alpha\beta$ mATP (10 μ M) (D). Data represented as mean \pm SEM (n=6).

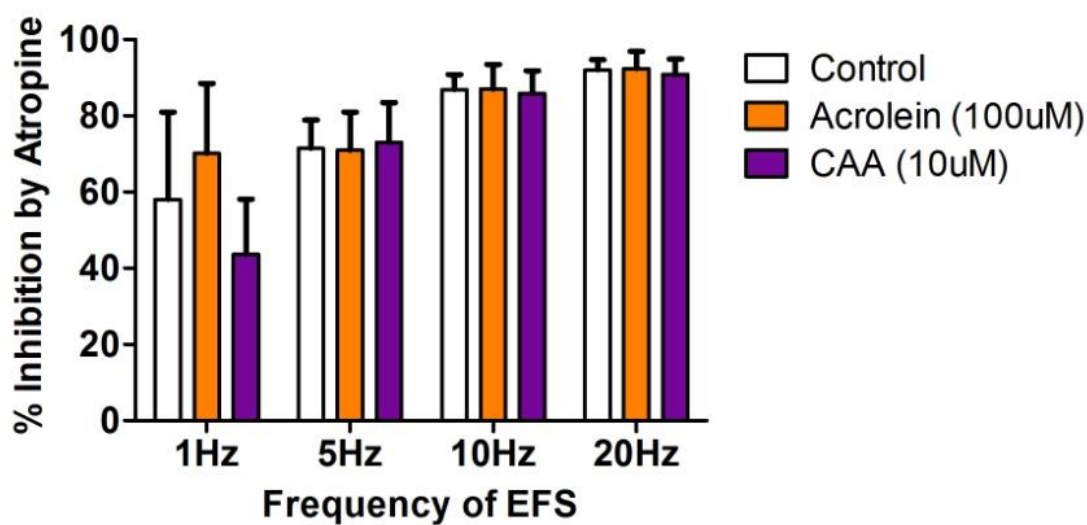


Figure 4.17: Percentage of inhibition of control response by atropine (1 μ M) in control, acrolein (100 μ M) and CAA (10 μ M) treated porcine detrusor strips in response to electrical field stimulation (40 volts, 1ms pulse-width, 5 second train). Data represented as mean \pm SEM (n=6).

4.4 DISCUSSION

This study aimed to investigate whether the CPO and IFO metabolites acrolein and CAA cause damage to full thickness bladder sections when applied on the luminal surface of the urothelium. To ensure viability of the tissues after treatment a period of four hours was used. This treatment time also correlates with the average time reported for the peak urinary concentrations of CPO and IFO metabolites to appear in the bladder (Koss, 1967, Takamoto et al., 2004).

Urothelial Effects of Acrolein or CAA

Both acrolein and CAA caused some damage to the urothelial layer of the pig bladder tissues and appeared to cause a reduction in the normal folding of the urothelial/LP layer. Intravesical instillation of acrolein in mice has caused similar results where doses of $>10\mu\text{g}/15\mu\text{L}$ resulted in urothelial erosion and oedema in the lamina propria sufficient to cause reduction of the urothelial folding within four hours (Bjorling et al., 2007, Batista et al., 2007). The damage caused by application of acrolein to the pig urothelium for four hours was not as severe as the damage observed in the previous mouse studies. This may be due to the fact that the pig bladder is thicker and more robust than the thin mouse bladder thereby preventing entry of acrolein into the urothelium or lamina propria for longer and delaying the onset of inflammation. Interestingly, the results of the present study show that CAA ($10\mu\text{M}$) is also capable of causing damage to full thickness bladder sections, although to a slightly lesser degree than acrolein ($100\mu\text{M}$). Although, this is unsurprising given that a 10-fold lower concentration of CAA was used. The thickness of the urothelium tended to decrease after treatment with acrolein or CAA indicative of death and loss of the urothelial cells. The urothelial thickness was not significantly different between control and treated tissues and caution should be taken when interpreting this result. However, given the consistent findings, the low error between repeated treatments and the small n-value the trend may be worth commenting on. Accordingly, a tendency toward a thinner urothelium after acrolein or CAA treatment provides some support to the findings of the previous Chapter wherein both metabolites were toxic to human urothelial cells. Furthermore, these results align with findings of previous studies discussed earlier wherein both acrolein and CAA have been found to be toxic to renal epithelial cells (Schwerdt et al., 2006, Broadhead et al., 1998). In addition, the current results do not rule out a role for CAA in the urotoxicity of CPO and IFO.

Next, the effect of acrolein or CAA on the function of the urothelium/LP was investigated by measuring the release of ATP, ACh and PGE_2 from urothelial/LP strips during basal and stretch

conditions. The levels of ATP, ACh or PGE₂ were unaffected by mechanical stretch of the tissues. Studies using a similar method have reported an increase in ATP levels induced by stretch of the porcine urothelium/LP (Sadananda et al., 2012, Smith et al., 2014) whereas there was no change in ACh levels after stretch (Smith et al., 2014). No studies have reported on stretch induced release of PGE₂ in porcine urothelium. Therefore, the results of the present study demonstrated similar ACh levels to previous findings however, the lack of stretch induced ATP release is unexpected. The ATP increase found by Smith et al., (2014) was modest and the samples were taken at an earlier time point in the study by Sadananda et al., (2012). Therefore, it is possible that stretch did induce an increase in ATP but that the later sampling point allowed for breakdown of the additional ATP.

In any event, pre-treatment with acrolein or CAA had no effect on the release of ATP, ACh or PGE₂ from the urothelium/LP. This was unexpected given the results in the previous Chapter reported increased mediator release from cultured urothelial cells. Furthermore, chronic treatment of rats with CPO has been shown to cause an increase in luminal ATP levels in response to hypo-osmotic stimulation of the urothelium (Smith et al., 2005). As discussed briefly in the previous Chapter, the initial effects of CPO treatment on rat urothelium include ulceration and erosion resulting in the loss of cells from the urothelium (Auge et al., 2013, Juszczak et al., 2010). The cells that survive start to express epidermal growth factor receptor (EGFR) and proliferate rapidly in order to repair the denuded areas (Romih et al., 2001). This rapid proliferation of cells results in urothelial hyperplasia, composed of undifferentiated cells (Boudes et al., 2011, Golubeva et al., 2014, Romih et al., 2001). After approximately a week of intense proliferation, differentiation of superficial cells begins and damaged cells are gradually removed by apoptosis until a normal three-layered urothelium is restored by two weeks following CPO treatment (Romih et al., 2001). Accordingly, the increased luminal ATP release observed by Smith et al., (2005) after three treatments of CPO is possibly due to a greater number of urothelial cells releasing ATP. The four hour incubations with acrolein or CAA caused the typical acute damage of urothelial erosion seen after CPO or IFO treatment. Accordingly, the current mediator results are actually higher than anticipated as the apparent urothelial cell loss after acrolein or CAA treatment would be expected to result in the release of fewer mediators. However, as demonstrated in the previous Chapter, the urothelial cells are able to release more ATP, ACh and PGE₂ per cell after acrolein or CAA treatment which may explain the fairly consistent mediator levels despite the loss of urothelial cells.

Therefore, the results of the present study demonstrated that both acrolein and CAA can induce urothelial damage after four hours resulting in urothelial cell loss. The results also suggest that

acrolein or CAA treatment may enhance mediator release from the urothelial cells, but in combination with urothelial cell loss this does not manifest as an increase in overall mediator levels. This supports the results of the previous Chapter showing that 24 hour treatment of urothelial cells caused a greater release of ATP, ACh and PGE₂ per cell. The urothelial damage observed in the present study may have been sufficient to cause loss of the barrier function of the urothelium allowing the toxic metabolites to penetrate the bladder wall and potentially alter detrusor function.

Effects of Acrolein or CAA on Contractility of Bladder Tissues

As shown above, both acrolein and CAA can cause urothelial damage when applied to the luminal side of full thickness porcine bladder. The urothelium can both increase or decrease contractile properties of the detrusor (Fry et al., 2010). In isolated bladder preparations, the nerve-mediated and carbachol-induced contractions were reduced in the presence of the urothelium (Hawthorn et al., 2000, Chaiyaprasithi et al., 2003). Similarly, the response of isolated rat bladder rings to ATP was lower in detrusor strips with intact urothelium (Khattab and Al-Hrasen, 2006). However, spontaneous contractile activity is increased in the presence of the urothelium (Kanai et al., 2007, Sui et al., 2008). This effect is thought to be due to waves of Ca²⁺ initiating in the urothelium or lamina propria which then propagate to the detrusor. Accordingly, damage to the urothelium/LP could result in changes to detrusor contractility and bladder function. In addition, the urothelium/LP is capable of contraction (Sadananda et al., 2008) and develops its own spontaneous contractile activity (Moro et al., 2011) and these functions could also be affected by acrolein or CAA induced damage. Furthermore, if the barrier function of the urothelium was damaged by acrolein or CAA treatment, the metabolites may also have damaged the detrusor itself. Accordingly, the contractility of the urothelium/LP, denuded and intact detrusor tissues was investigated after four hour acrolein or CAA incubation.

Firstly, the maximum response of the tissues to KCl, ATP and carbachol were determined. All three control tissue types contracted in response to the agonists with the detrusor generally producing a greater response than the urothelium/LP or the intact tissues. In the urothelial/LP strips, treatment with acrolein or CAA had no significant effect on the contractions to KCl, ATP or carbachol. However, the detrusor strips tended to have reduced contractile responses to all three agents after acrolein or CAA treatment. This trend was not statistically significant however it suggests that some functional changes may be occurring after treatment. Previous studies have reported reduced detrusor contractions in response to carbachol and/or ATP after treatment with CPO or IFO (Mok et al., 2000, Macedo et al., 2011, Giglio et al., 2005,

Andersson et al., 2008, Giglio et al., 2007). However, most of these studies also demonstrated a similar reduction in tissue contraction to KCl. The reduced KCl response indicates a detrimental effect to the general contractility of the tissue after treatment rather than a change in the receptor mediated responses. Therefore, it appears that the detrusor may have sustained some minor damage during the treatments with acrolein or CAA which could be due to direct injury by the metabolites entering the bladder wall through a damaged urothelial barrier, or could be the result of an inflammatory response in the tissues initiated by the urothelial damage.

In the presence of the urothelium/LP the control detrusor responses to KCl, carbachol and ATP were lower than the denuded detrusor responses. As discussed previously, in response to muscarinic receptor stimulation the urothelium releases a diffusible factor (UDIF) which inhibits the contraction of the underlying detrusor (Hawthorn et al., 2000) while an inhibitory effect of the urothelium has also been shown in response to ATP (Khattab and Al-Hrasen, 2006). The presence of the urothelium/LP reduced responses of the detrusor by approximately 50% in control tissues, which is in line with the effect of UDIF or the urothelial inhibition reported these previous studies.

The inhibitory effect of the urothelium/LP on detrusor contractions to KCl was unaffected by acrolein or CAA pre-treatment. However, the urothelial/LP inhibition of detrusor contractions to ACh and ATP was less after pre-treatment with acrolein or CAA. The responses to the agonist carbachol were reduced by only 30% in treated intact tissues indicating a loss of the muscarinic mediated urothelial inhibitory effect after treatment. The responses to ATP were not inhibited by the presence of the urothelium/LP suggesting a complete loss of the purinergic mediated urothelial inhibition. A similar result has been reported in porcine tissues treated with DMSO where the inhibitory effect of the urothelium/LP was reduced significantly after treatment (Smith et al., 2014). Accordingly, it appears that the inhibition of detrusor contraction by the urothelium/LP is reduced after acrolein or CAA treatment.

Comparable results were also obtained when full concentration-response curves to carbachol were performed in urothelial/LP, denuded detrusor and intact strips. The concentration-response curves were almost identical in control and treated urothelial/LP and intact strips. However, the denuded detrusor strips tended to have decreased contractile responses over the entire curve. Furthermore, the control detrusor responses to carbachol were significantly lower in the presence of the urothelium whereas this effect was reduced after acrolein or CAA treatment. This further supports a loss of the inhibitory effect of urothelial/LP after acrolein or CAA treatment. The responses of treated and control tissues, when

represented as a percentage of maximum, were almost identical and the pEC₅₀ values of carbachol were unchanged after treatment.

Next the ability of the tissue to relax in response to cumulative concentrations of isoprenaline (β -AR agonist) was investigated. The urothelial tissues relaxed in response to isoprenaline and the responses were unchanged by previous treatment with acrolein or CAA. The acrolein or CAA pre-treated detrusor strips were slightly less responsive to isoprenaline whereas the responses of treated intact strips were the same as controls. Previous studies have reported that three days after CPO treatment (100mg/kg) isolated rat bladder strips had inhibited relaxations to isoprenaline (Giglio et al., 2007, Vesela et al., 2012). The immediate effects of acrolein or CAA treatment in the present study, although not statistically significant, support a loss of relaxation response after exposure to CPO or IFO. Again, it appears that acrolein and CAA have a general detrimental effect on muscle function. Furthermore, a reduced ability of the bladder to relax during filling could contribute to storage symptoms such as reduced bladder capacity and increased urinary frequency.

The spontaneous contractions of the detrusor and urothelium/LP were also investigated to determine whether acrolein or CAA alter the frequency or amplitude of these contractions. Both frequency and amplitude of the spontaneous contractions were significantly higher in control urothelial/LP tissues than in detrusor tissues (**Figure 4.13**). This supports the findings reported by Moro et al., (2011) that porcine detrusor muscle had a significantly lower rate of spontaneous contractions than the urothelium/LP. Treatment with acrolein or CAA had no significant effect on the average frequency or the amplitude of spontaneous contractions in the urothelium/LP or the detrusor compared to control tissues. This was unexpected given that cystometry in rats treated with CPO have shown enhanced spontaneous activity of the bladder during filling (Pan et al., 2012). However, this may represent a difference between *in vivo* and *in vitro* tissue responses. In isolated bladder strips from mice treated with CPO, 33% of the tissues exhibited spontaneous contractile activity, while the control tissues failed to show any spontaneous contractions (Okinami et al., 2014). As discussed further below, the metabolism of CPO and IFO *in vivo* is likely to cause toxic metabolites to be present in the urine for up to 24 hours. Accordingly, the increased spontaneous activity seen in the previous studies may have been due to the damaging metabolites being present in the urine for longer than the four hours used in this study. Furthermore, the inflammatory response may have been greater in the *in vivo* models used in these studies causing greater damage and affecting the bladder function to a greater extent than in the isolated bladder treatment used in the present study. However, the

results of this Chapter show that acute, four hour acrolein or CAA treatment of isolated bladder does not affect the spontaneous activity of the detrusor or urothelium/LP.

In the present study, an impaired urothelial inhibitory effect appears to cancel out the reduced detrusor contractility after acrolein or CAA treatment producing a similar result to controls in the intact tissues. The implications of these changes are not clear, although both acrolein and CAA appeared to have similar effects and it may be possible that both metabolites contribute to urotoxicity of CPO and IFO.

Effects of Acrolein or CAA on Efferent Nerve Mediated Responses of the Bladder

There appears to be some reduction in contractility of the detrusor to endogenous agents after acrolein or CAA treatment however, it is the activity of the efferent nerves that would be controlling responses in physiological conditions. Therefore, the EFS induced efferent nerve responses were investigated. The EFS parameters used in the present study caused frequency dependent contractions that were sensitive to TTx confirming that they were nerve induced.

In control tissues, the inhibition of NO production with L-NNA did not affect the nerve mediated responses. NOS has been identified in nerve fibres in the bladder (Andersson and Persson, 1995) however, the detrusor has a low sensitivity to NO, making it unlikely to have a relaxatory role in normal detrusor (Andersson and Arner, 2004). Similar results have been demonstrated in human and rat detrusor tissues wherein NOS inhibition did not significantly enhance EFS induced contractions (Ehren et al., 1994b, Persson et al., 1992, Vesela et al., 2012). Accordingly, the lack of effect of L-NNA in the present study is not surprising and supports that neuronal NO has little effect on normal detrusor contraction.

Next, blocking the muscarinic receptors with atropine almost completely abolished the EFS induced contractions in the porcine detrusor. As discussed earlier, human bladder nerve mediated contraction is almost completely due to ACh (Chess-Williams, 2002, Fetscher et al., 2002), while a NANC portion of nerve induced responses appears in disease and is thought to be due to ATP (Palea et al., 1993). In pig bladder ACh contributes more at high frequencies of nerve stimulation than low frequencies making up 83% of the response at 50Hz (Sibley, 1984). Given that pig bladder contracts to ATP the remaining non-cholinergic response is also thought to be due to ATP (Inoue and Brading, 1991). Similar results were observed in the present study wherein atropine had a greater effect at high frequencies than low frequencies contributing to 90% of the EFS induced response at 20Hz. In addition, desensitisation of purinergic receptors with

$\alpha\beta$ mATP appeared to partially reduce the contraction at low frequencies but not high frequencies. This suggests that ATP and ACh may contribute to EFS induced responses in a combination that changes depending on frequency of nerve stimulation. Specifically, the ATP portion of the EFS responses appears greatest at low frequencies of stimulation and reduces as frequency increases while a simultaneous increase in ACh contribution is observed. However, in this work, the effect of $\alpha\beta$ mATP was small and not statistically significant which suggests that ATP may not have been contributing to nerve mediated contractions or that a small population of purinergic receptors insensitive to desensitisation by $\alpha\beta$ mATP may have been present.

Treatment with acrolein (100 μ M) or CAA (10 μ M) had no significant effect on the response to EFS. Furthermore, no change in the contribution of NO, ACh, or ATP was seen after treatment with acrolein (100 μ M) or CAA (10 μ M). This supports previous findings that CPO treatment does not affect the contribution of ACh or ATP to nerve induced contractions. For example, Mok et al., (2000) found that EFS induced contractions in isolated bladder strips from CPO treated *Suncus murinus* (shrew) were the same as in control strips and atropine and $\alpha\beta$ mATP reduced responses to a similar extent in both groups. Another study in rats also found that atropine and $\alpha\beta$ mATP reduced responses to a similar extent in control and CPO treated bladder strips (Giglio et al., 2007). However, three days after CPO treatment, Vesela et al., (2012) demonstrated that inhibition of NO production slightly enhanced EFS induced contractions in CPO treated bladder strips but not in control strips. Previous studies have suggested that the urothelium of CPO treated rats (but not control rats) releases NO in response to muscarinic receptor stimulation reducing the associated detrusor contraction (Andersson et al., 2008). This mechanism may have contributed to the increased nerve mediated contraction seen by Vesela et al., (2012) after blocking NO production. However, these effects were seen three days after administration of CPO whereas the present study investigated acute effects of acrolein and CAA which do not appear to change NO contribution to EFS induced responses.

Relevance of the Treatment Period

Although the peak urinary concentrations have been reported to appear in urine of patients four hours after treatment with CPO or IFO (Koss, 1967, Takamoto et al., 2004), the metabolites can continue to be excreted in the urine for up to 24 hours after treatment (Bagley et al., 1973, Jardine et al., 1978, Sladek et al., 1980, Joqueviel et al., 1997). Accordingly, it is possible that the bladder would be exposed to these metabolites for a longer period than four hours. Granted, the concentrations would be varying over time as the metabolites are cleared from the circulation. Accordingly, it is possible that the treatment period of four hours was not sufficient

to imitate the conditions of the bladder after CPO or IFO treatment. However, given the limited timeframe in which isolated tissues can be used, four hours was a feasible treatment time that could be used as a baseline for further investigations. The ideal model of a clinical setting is to treat an animal and allow normal drug metabolism and excretion to produce cystitis similar to what patients would experience.

Conclusions

The results of the present study have demonstrated that both acrolein and CAA are capable of causing some urothelial damage within just four hours. This supports the findings of the previous Chapter suggesting that CAA and not just acrolein may contribute to the urotoxicity of CPO and IFO. The damage induced by both acrolein and CAA suggest a loss of detrusor muscle function, but no change in efferent nerve induced responses. This also supports findings of systemic CPO treatment in animals showing a significant reduction in detrusor contractility several days after treatment and suggests that these effects may start as early as four hours after metabolite excretion. Acrolein and CAA treatment also tended to cause reduction in the inhibitory effect of the urothelium on the detrusor. Whether this effect persists at the later stage of CPO or IFO induced cystitis is unknown but it may contribute to the bladder overactivity experienced by patients. CAA and not just acrolein may have a role in the urotoxicity of CPO and IFO and ignoring its role may be contributing to the limited efficacy of preventative measures. Accordingly, this research suggests that CAA production should be considered when trying to prevent cystitis in patients receiving CPO or IFO.

CHAPTER 5: Effects of Systemic Cyclophosphamide or Ifosfamide on Bladder Sensory Nerve Activity in Mice

5.1 INTRODUCTION

As covered previously, bladder afferent nerves sense chemical and mechanical changes in the bladder and relay information about volume during storage and the amplitude of contractions during voiding to the central nervous system (Kanai and Andersson, 2010). The activity of the afferent nerves controls the storage and micturition reflexes of the bladder and is imperative to normal function of the bladder.

Classification & function of afferent nerves

There are two main types of afferent fibres in the bladder, myelinated A- δ fibres and the more common unmyelinated C-fibres (Kanai and Andersson, 2010, Birder et al., 2010a, Gabella and Davis, 1998). Afferent nerves can be classified according to a variety of different characteristics. However, one of the most frequently used classifications is whether the fibres become active at low (<15mmHg) or high (>15mmHg) intravesical mechanical thresholds (Daly et al., 2007, Rong et al., 2002, Shea et al., 2000). Nerves that do not respond to mechanical stimuli are termed silent afferents and may become mechanosensitive during inflammation (Shea et al., 2000). It is estimated that approximately 75% of mouse pelvic afferents are sensitive to stretch with receptive fields throughout the bladder (Jung et al., 2012). Recently, afferent fibres in the mouse have been further classified on the location of their receptive field and responses to various mechanical stimuli (urothelial stroking, blunt probing and stretch) with four distinct populations being observed (muscular, urothelial, muscular/urothelial, and serosal) (Jung et al., 2012). A more detailed description of each classification follows:

Urothelial: Afferents that responded to fine stroking of the urothelium but not to bladder stretch were classified as urothelial afferents. Urothelial afferents made up 9% of the pelvic nerve fibres.

Muscular: Muscular afferents were characterised as having receptive fields in the bladder wall and responded to probing and stretch but not urothelial stroking. Muscular fibres were the most common pelvic nerves fibres making up 63% of the sample.

Muscular/urothelial: Afferents that responded to both urothelial stroking and stretch of the bladder were characterised as urothelial/muscular fibres. Of the total sample of pelvic fibres, 14% were classified as muscular/urothelial fibres.

Serosal: Afferent fibres with receptive fields in the bladder wall that responded to probing but not to stretch or urothelial stroking were deemed to be serosal afferents. This group also made up 14% of the pelvic fibres.

Similar classification of afferent nerves has been made in other species such as the guinea pig. Zagorodnyuk et al., (2006, 2007) identified four classes of afferents including stretch-sensitive muscle and muscle/mucosal mechanoreceptors as well as stretch-insensitive mucosal mechanoreceptors and chemoreceptors. Firstly, the muscle mechanoreceptors responded to stretch but not mucosal stroking, which is similar to the muscular afferents described in mice. Secondly, the muscle/mucosal mechanoreceptors responded to stretch and mucosal stroking, a similar response to the muscular/urothelial afferents described by (Jung et al., 2012). The third group of afferents termed mucosal mechanoreceptors responded to mucosal stroking but not stretch, which is similar to the urothelial afferents in mice. Lastly the chemoreceptors were stimulated by acid or high K^+ but not stretch or stroking.

It was originally postulated that mechanoreceptors are in series tension receptors that fire in proportion to intravesical pressure during both contraction and distension (Iggo, 1955). However, two response patterns of stretch sensitive bladder afferent nerves have recently been reported in rats (Shea et al., 2000). One type demonstrated continuously increasing firing rates as the bladder filled to maximum distension, while the second type exhibited peak firing rates at lower than maximum pressure during filling followed by a plateau or a decrease in activity at higher pressures. These two response patterns have also been shown in mice (Jung et al., 2012) and were termed encoding (firing increasing to maximum stretch) and non-encoding fibres (peaking at lower than maximum stretch followed by a plateau). The non-encoding fibres were found to be more common than encoding fibres.

The ability of afferent nerves to sense a mechanical force may be due to direct and indirect mechanisms. Direct mechanisms of mechanotransduction include the opening of gated ion channels by deformation of the channel or by tugging on the channel protein by cytoskeletal or extracellular tethers (Gillespie and Walker, 2001, Hamill and Martinac, 2001). Indirect mechanotransduction may also occur due to the release of mediators from the urothelium in response to stretch or via electrical communication from the interstitial cells (Cockayne et al., 2000, Fry et al., 2007).

Several studies have demonstrated the ability of afferent fibres to become sensitised to irritation or inflammation (Rong et al., 2002, Jung et al., 2012, Habler et al., 1990). An increase in

afferent nerve response magnitude and a reduction in response threshold are features of afferent sensitisation (Bessou and Perl, 1969). Sensitisation can result in an increase in the excitability of the receptive ending to stimuli and is thought to be important in the development of hyperalgesia/hypersensitivity (Bessou and Perl, 1969). Rong et al., (2002) demonstrated in mice that silent fibres could be sensitised by intravesical instillation of $\alpha\beta$ mATP and become mechanosensitive. Interestingly, nearly all of the high threshold fibres were also sensitised by $\alpha\beta$ mATP demonstrating a lower threshold of activation and increased peak activity during distension. Approximately half of the low threshold fibres were also sensitised by the agonist, reaching a higher peak of activity while their activation threshold remained the same. Similar results were reported by Xu & Gebhart (2012) using an inflammatory mixture of bradykinin, serotonin, histamine and PGE₂ in mice. Results showed that both high and low threshold fibres were sensitised by the inflammatory mixture resulting in a greater peak firing response. In addition, the activation threshold of the high-threshold fibres decreased to be similar to that of the low-threshold fibres.

The effect of CPO or IFO on afferent nerves in the rodent

Treatment with CPO or IFO causes bladder inflammation (cystitis) and can result in sensory disturbances such as pain, feelings of residual volume or urinary frequency. Similar functional changes which are suggestive of hyperreflexia have been reported in animals treated with CPO or IFO (Okinami et al., 2014, Macedo et al., 2011, Wang et al., 2008b, Kageyama et al., 2008, Andersson et al., 2008, Ito et al., 2008, Juszczak et al., 2009) and even after intravesical instillation with acrolein (Wang et al., 2008b). However, few studies have looked into the direct effects of CPO or IFO treatment on afferent nerve function and responsiveness. Whole-cell patch-clamp recordings of isolated DRG neurones from CPO treated rats demonstrated lower thresholds for spike activation and subsequently more action potentials to a current stimulus compared to controls (Yoshimura and de Groat, 1999). Dang et al., (2008) using the same model, reported similar results but additionally found that responses to purinergic receptor agonists were increased in DRG neurones from CPO treated rats. Multiunit nerve recording in rats demonstrated that CPO treatment caused increased activity of the pelvic nerve afferents during bladder distension (Yu and de Groat, 2008). At low intravesical pressure, the enhanced nerve firing could be reduced by the P2 receptor antagonist PPADS. In addition, the threshold of activation of pelvic nerves to electrical stimulation was also lower in CPO treated rats.

These studies provide evidence that afferent activity is altered after CPO treatment however it remains to be shown whether low or high threshold nerves are affected by treatment and

whether their responses to other agonists are changed. In addition, no studies to date have investigated the direct effect of IFO on bladder afferent firing.

Evaluation of nerve function

Measuring the characteristics and excitability of isolated DRG neurones is often used as a preliminary way to study nerve function. This method is limited as it does not provide a link to physiological conditions in the bladder such as pressure during filling or detrusor tone. A pelvic nerve preparation has been used previously to study the function of the afferent nerves in relation to bladder distension in mice (Rong et al., 2002, Daly et al., 2007). The model maintains the whole urinary tract intact from kidney to urethra and it remains attached to the surrounding tissues. A catheter inserted through the urethra allows filling of the bladder while a two-way catheter secured in the dome of the bladder allows emptying of the bladder and measurement of intravesical pressure by a pressure transducer. The pelvic nerves can be dissected into fine branches and nerve impulses recorded from a branch using a suction electrode. This model allows recording of afferent activity in response to repeated distension of the bladder or application of pharmaceutical agents. In addition, the preparation enables discrimination of single afferent fibres in the nerve branch and classification of each fibre into a low or high threshold category. This model allows more functional investigation of bladder and nerve interactions and was used to test the aims of the present study.

Aims

The aim of the present study was to investigate the activity of pelvic afferent nerves of the mouse bladder after treatment with CPO or IFO. Specific aims were:

- To determine the effect of CPO or IFO treatment on total afferent nerve activity during bladder distension;
- To investigate the effect of CPO or IFO treatment on the activity of low and high threshold afferent nerve fibres;
- To assess the activity of afferent nerves in response to various agonists and whether the responses are altered after CPO or IFO treatment;
- To determine the contribution of muscle tone to afferent nerve responses and whether it changes after CPO or IFO treatment.

5.2 METHODS

Animals

Young adult male C57/6B mice were obtained from the Griffith University Gold Coast animal house under ethics licence No. MSC/06/13 held by Dr. Roselyn Rose'Meyer. Animal treatments and euthanasia were performed at the Griffith University animal house and tissues were transported to Bond University for all experimental procedures (Bond University ethics No. RO1762). The C57/6B is a widely used inbred strain of mouse. Three to five month old (young adult) male mice were used for this project. The animals were housed for at least one week in a controlled environment with 12 hour light/dark cycles, constant temperature maintained to $\approx 23^{\circ}\text{C}$ with free access to food and water. An animal was selected for treatment in one of three groups: control (saline treated), CPO (100mg/kg) treated or IFO (200mg/kg) treated.

Several studies have used CPO or IFO to treat cancer in mice and the reported doses range between 30mg/kg – 200 mg/kg intraperitoneally for CPO (Kusnierczyk et al., 1999, Harnack et al., 2011, Ma et al., 2011) and 60 - 350 mg/kg intraperitoneally for IFO (Silbermann et al., 1990, Brucker and Sieg, 1999, Wang et al., 2008a, Yamada et al., 2009). For this study, a CPO dose of 100mg/kg and an IFO dose of 200mg/kg were chosen which fall within the doses used for cancer treatment in mice. Both drugs were dissolved in saline and injected intraperitoneally while, control animals were treated with an equal volume of saline. Animals were kept separately after treatment and were monitored for signs of distress or pain at 6 and 12 hours post treatment. Animals were sacrificed humanely 24 hours after treatment by cervical dislocation in accordance with ethical committee approval and the guidelines of the National Health and Medical Research Council of Australia. An incision was then made in the abdomen of the animal removing the skin and peritoneum of the abdomen. The gastrointestinal tract superior to the bladder was removed revealing the kidneys. The vertebra of the animal was severed above the kidneys at approximately the level of L2. The tail and the hind limbs were removed and the remaining pelvic area was placed into cold Krebs solution (recipe page on 53) for transport.

Afferent Nerve Recording

After dissection, the lower abdomen was secured in a 30mL bath and was continually superfused with Krebs solution gassed with 95% O₂ - 5% CO₂ at a rate of 6mL/min and was maintained at a constant temperature of 35°C. Using a dissection microscope (WPI, PZMIII), the testes, prostate and surrounding connective tissues were dissected away from the bladder exposing the pubic symphysis. The ureters were ligated to prevent leakage of intravesical fluid. The pubic symphysis was cut either side of the urethra and was removed. The pelvic bone on either side of the urethra was also removed along with remaining bone from the hind legs. The end of the urethra was cut and a catheter (OD 0.03IN) attached to a syringe pump (Genie, Kent, multi-phaserTM model NE-1000) was inserted into the bladder and secured in place with silk suture to allow distension with isotonic saline (0.9%) solution. The bladder was then partially filled to allow the dome to be pierced with a fine syringe needle (BD microlanceTM, 19G). A two way catheter was inserted into the dome of the bladder to allow measurement of the intravesical pressure via a pressure transducer (DTXTM plus DT-XX, Becton Dickinson, Singapore) and to allow emptying of its contents via a two-way outflow tap (see **Figure 5.1**). The two-way catheter was secured inside the bladder using silk suture and the position of the tap (open or closed) allowed control over filling or emptying of the bladder.

The bladder was distended by infusing saline at 30µl/min to a maximum intravesical pressure of 40mmHg to check the viability of the catheterisation. If leaks were apparent, then all suture ties were checked and connections in the catheter lines tightened. The infusion pump was stopped and the tap in the two way catheter was opened to allow complete evacuation of the intravesical contents of the bladder.

Following successful catheterisation, bladder nerve bundles were identified. Bladder nerve bundles converge with urethral bundles as they both travel toward the spinal cord and it is at this meeting point that they are easiest to identify under the microscope. Firstly, connective and fatty tissues were removed from the distal end of the urethra toward the bladder until the urethral nerve bundles were identified. Then further tissue was removed until the urethral bundles join the bladder bundles and until an individual nerve branch could be divided from the bladder bundle. The nerve branch was then inserted into a suction electrode (tip diameter 25-50µm) attached to a Neurolog headstage (NL100, Digitimer Ltd, UK) to allow recording of multi-unit afferent nerve activity (**Figure 5.2**). The NL100 was connected to an AC pre-amp (NL014, Digitimer Ltd) which amplified the nerve signals (x10,000) before they were filtered (NL125, band pass filter, Digitimer Ltd) and passed through a 50/60Hz electrical noise

eliminator (Humbug, Quest Scientific, Canada) to a Micro1401 analogue to digital interface (Cambridge Electronic Design, UK) and then visualised on a computer using Spike2 software (version 7.1, Cambridge Electronic Design, UK). Whole nerve multi-fibre afferent nerve activity was quantified using the Spike2 software which counted the number of action potentials crossing a pre-set threshold. The bladder was again distended at 30 μ l/min to 40mmHg to ensure the nerves isolated originated in the bladder and if no signal was obtained, another nerve branch was isolated and placed in the suction electrode.

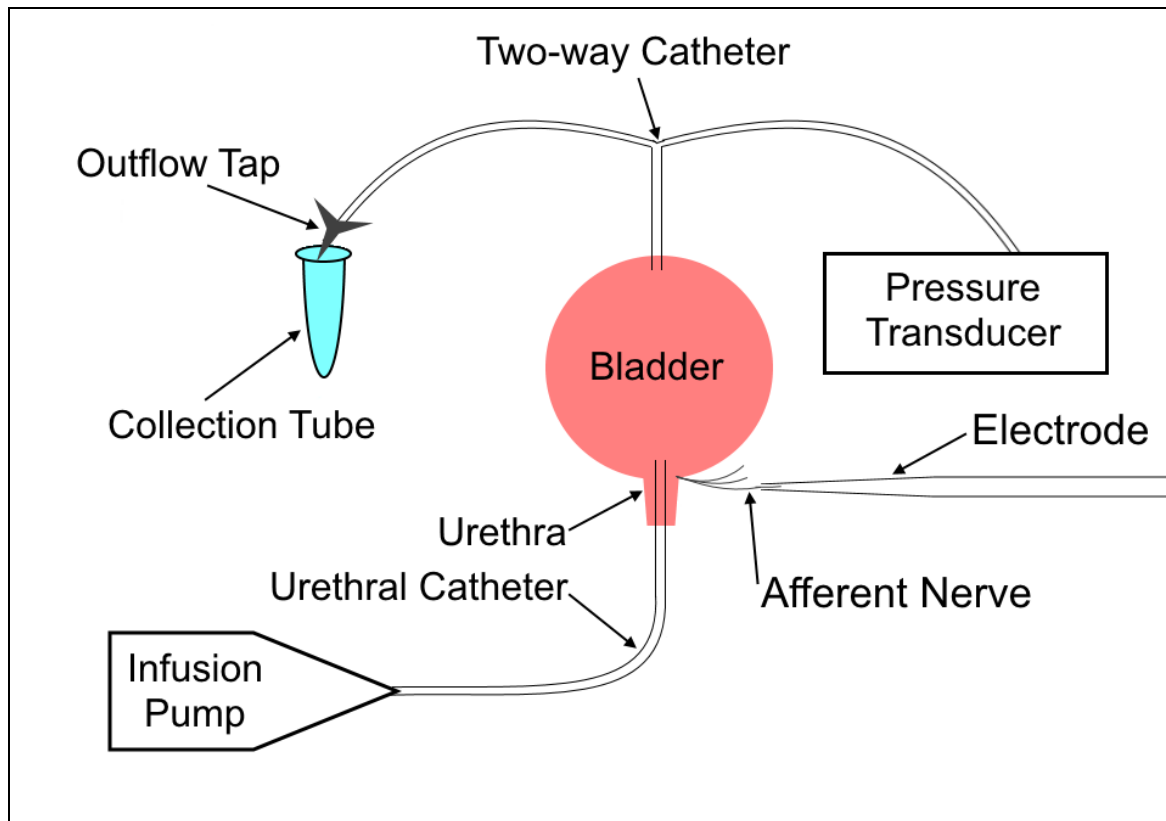


Figure 5.1: Schematic showing the mouse afferent nerve recording set up with the bladder connected to a urethral catheter and a two-way catheter.

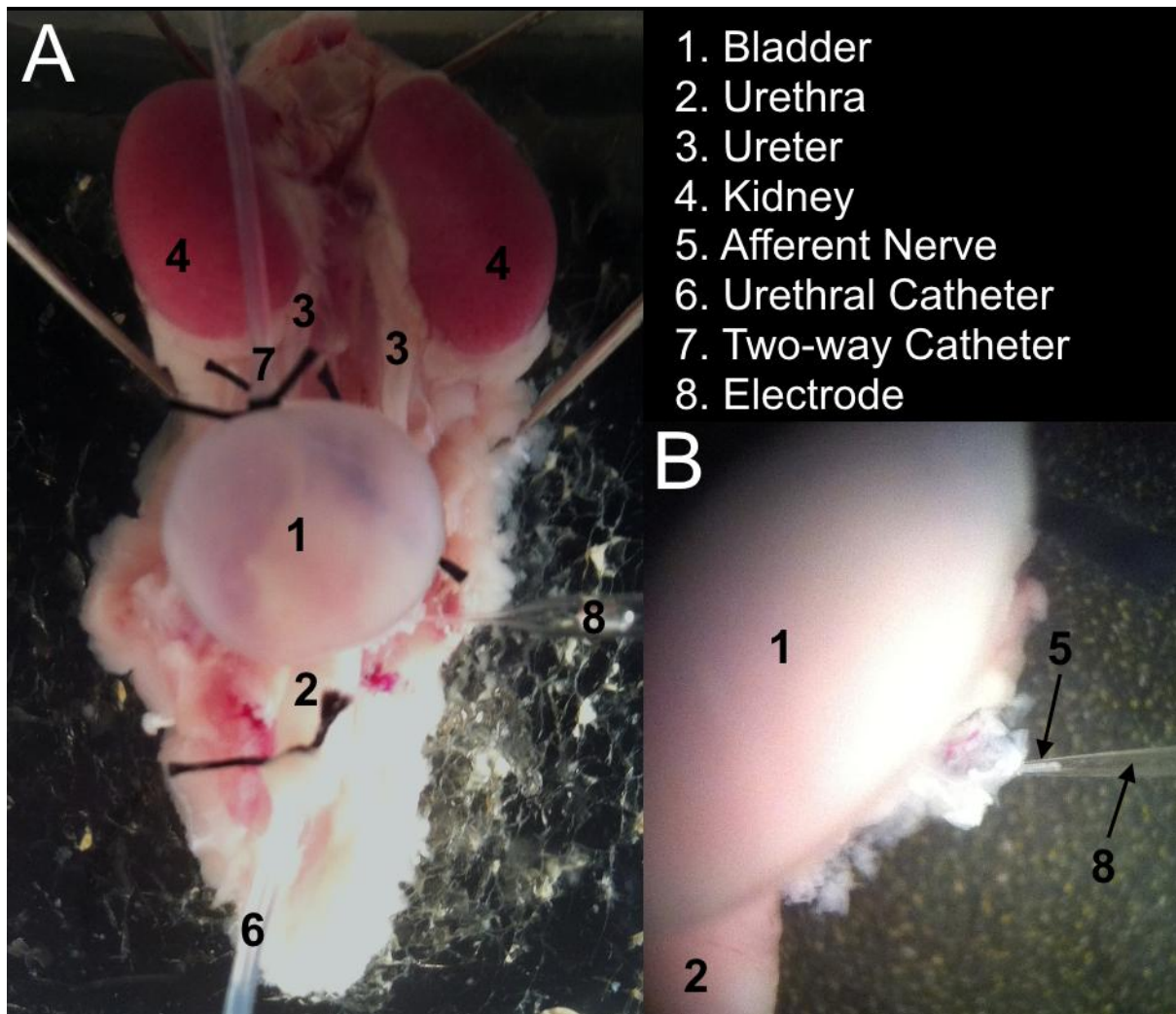


Figure 5.2: Photograph showing the mouse afferent nerve recording set up with the bladder connected to a urethral catheter and a two-way catheter (A) and a closer photograph showing the nerve branch inside the suction electrode (B).

Intravesical Pressure & Bladder Distensions

An infusion pump was used to pump isotonic 0.9% saline solution into the bladder at a constant rate of 30 μ l/min. When the outflow tap was in the closed position the bladder filled with saline and intravesical pressure developed. This pressure was measured via the pressure transducer in mm of mercury (Hg). The bladder and catheter set up created a small measurable pressure reading even when the bladder was empty. This baseline pressure was subtracted from the pressures during distension to give the change in pressure.

Typically, the bladder distensions performed during the following experiments were to a maximum of 40mmHg. As volume in the bladder increased, intravesical tension also increased. Bladder filling typically included two distinct phases (**Figure 5.3-A**). The initial phase was characterised by a gentle increase in pressure to approximately 15mmHg during which time the bladder accommodated a large volume of saline. This was followed by a second phase

characterised by a steep increase in pressure up to 40mmHg in response to a small volume. The bladders showed stable and repeatable pressure responses to distension at 30 μ l/min (Figure 5.3-B).

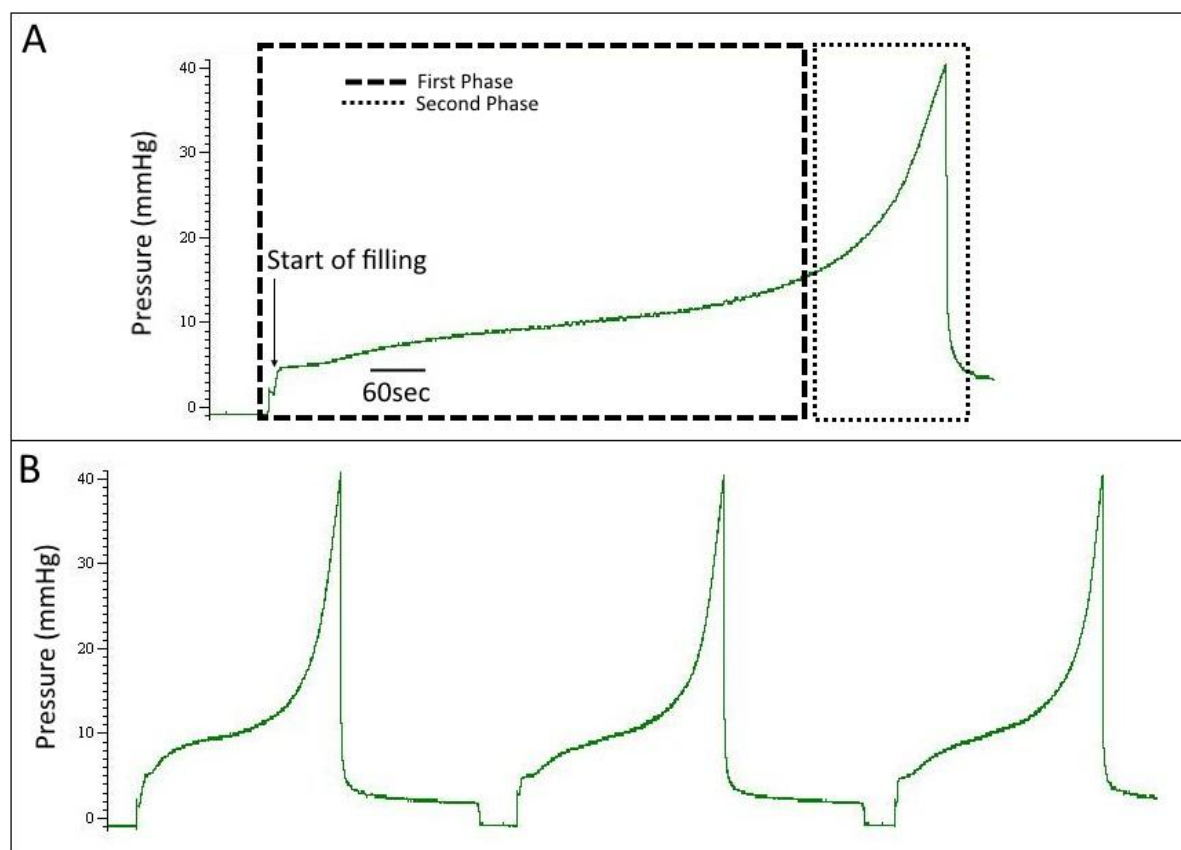


Figure 5.3: Representative trace of distensions to increasing intravesical pressure to 40mmHg in a mouse bladder showing the two phases of filling (A) and the repeatability of the distension response (B).

Mediator Release

Once the preparation had been successfully set up, samples were collected for measurement of basal and stretch release of ATP and ACh. To collect basal samples, the outflow tap was opened and the infusion pump turned on to infuse saline through the bladder at 30 μ l/min with no pressure development. The pump was left to run for nine minutes in total, the first two minutes were to flush the bladder and the saline flowing from the outflow tap during this time was discarded. For the remaining seven minutes the saline passing through the bladder was collected in a tube on ice and transferred to storage at -20°C as soon as possible after collection for later analysis. A volume of 210 μ l was collected during the seven minutes which was a sufficient amount for analysis of ATP and ACh.

Next, to collect samples during bladder distension (stretch) the outflow tap was closed and the infusion pump filled the bladder at 30 μ l/min. Once the intravesical pressure reached 20mmHg,

the infusion pump was turned off and the outflow tap opened. The luminal contents of the bladder were collected into a tube on ice and were stored as soon as possible after collection at -20°C. This process was repeated twice more in the presence of the ATPase inhibitor ARL67156 (100µM) in saline, with 10 minutes recovery between each distension.

The basal and stretch samples with and without ARL67156 were then used within three weeks in the assays described in Chapter 2 for quantification of ATP and ACh levels.

Nerve Firing

Multi unit nerve activity was quantified using Spike2 software which counted the number of action potentials or 'spikes' that cross a pre-set threshold (**Figure 5.4-C**) and simultaneously represents the nerve activity per one second bin in a sequential rate histogram (nerve impulses per second [imp/sec]) (**Figure 5.4-B**). As the bladder fills, the electrical impulses increase and translate as larger spikes which then cross the threshold registering as a nerve impulse (**Figure 5.4-D & E**). The threshold can be manually set depending on the parameters to be measured. In the following experiments, the aim was to measure total nerve activity. Accordingly, the threshold was set to measure nerve activity developed by an empty bladder. Baseline afferent activity was obtained by averaging the discharge in the 100 second period prior to the distension. The afferent nerve response during bladder distension was calculated by measuring the afferent activity (imp/sec) at each intravesical pressure increment up to 40mmHg. These values were then subtracted from the baseline firing to give a total change in afferent nerve response during distension.

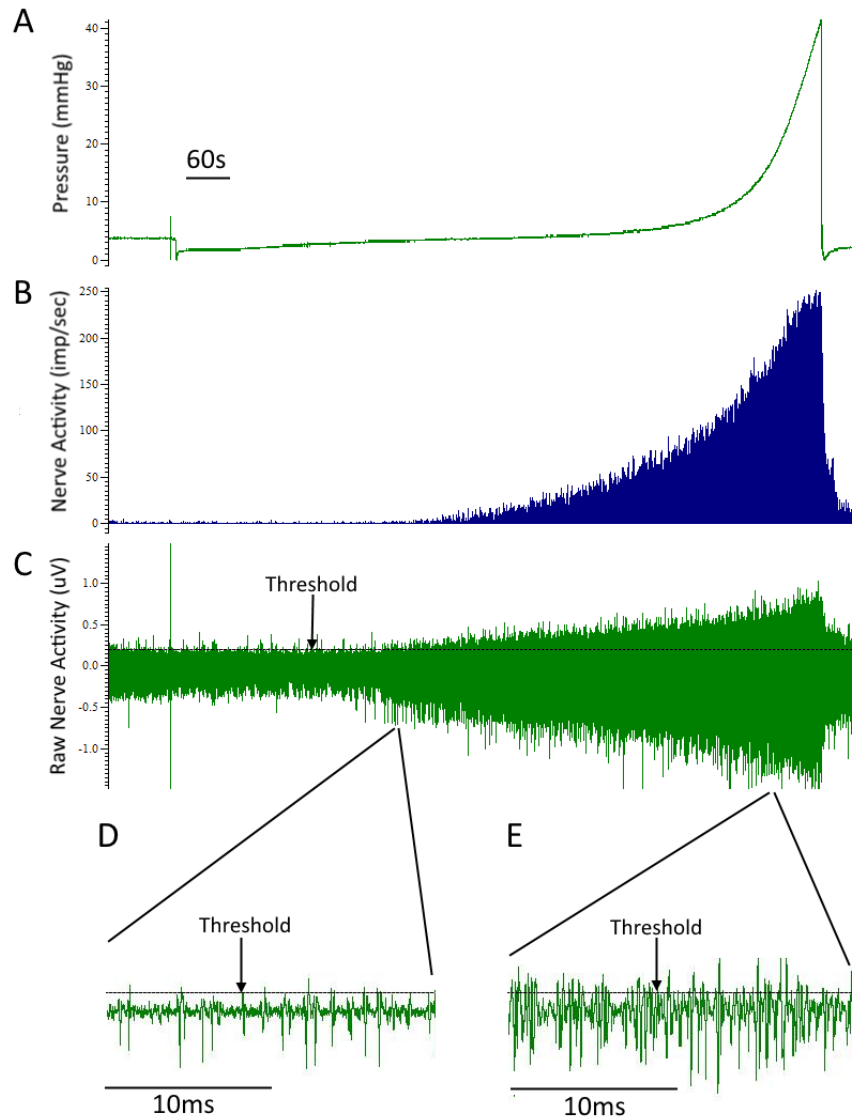


Figure 5.4: Representative traces during distension of a mouse bladder showing intravesical pressure (A), a sequential rate histogram of the number of nerve impulses per second (B) generated from raw nerve activity (C) when action potentials cross a pre-set threshold. A closer view of the raw nerve activity during low intravesical pressure (D) and high intravesical pressure (E).

Compliance

Bladder compliance is the relationship between bladder volume and bladder pressure or more simply, the ability of the bladder to accommodate a volume. In the following experiments, bladders were filled to a consistent pressure. This ensured the bladders received the same mechanical stimulus and reduced variability due to bladder size. As bladders were filled at a constant rate, volume could be calculated from time since the start of distension using the following equation:

$$\text{Volume } (\mu\text{l}) = \text{Rate } (\mu\text{l/sec}) \times \text{Time (sec)}$$

The ability of the bladder to accommodate a set intravesical pressure was also used as a measurement of compliance. In the following experiments the bladders were filled to a set pressure of 20mmHg and left for up to an hour for bladder pressure to reach a stable plateau. Changes in intravesical pressure and afferent activity were measured during filling and accommodation and bladder compliance was represented as an XY graph showing the relationship between volume and pressure in each setting.

Spontaneous Contractions

During bladder distension and accommodation, phasic intravesical pressure increases were observed (**Figure 5.5** and **Figure 5.6**). These spontaneous contractions demonstrated consistent frequency and amplitude within a bladder preparation and were associated with phasic increases in afferent nerve activity. The frequency and amplitude of spontaneous contractions during filling and accommodation were also quantified. Spontaneous contractions during filling were measured during the 200s preceding intravesical pressure reaching 15mmHg and during accommodation were measured during the 200s at the end of the 1 hour stabilisation period. Amplitude of a contraction was measured by subtracting the pressure at the beginning of the contraction from the pressure at the peak of the contraction and the average represented as mmHg. Average frequency was represented as contractions per minute. The increase in afferent activity associated with each contraction was also quantified and the average represented as nerve impulses per second.

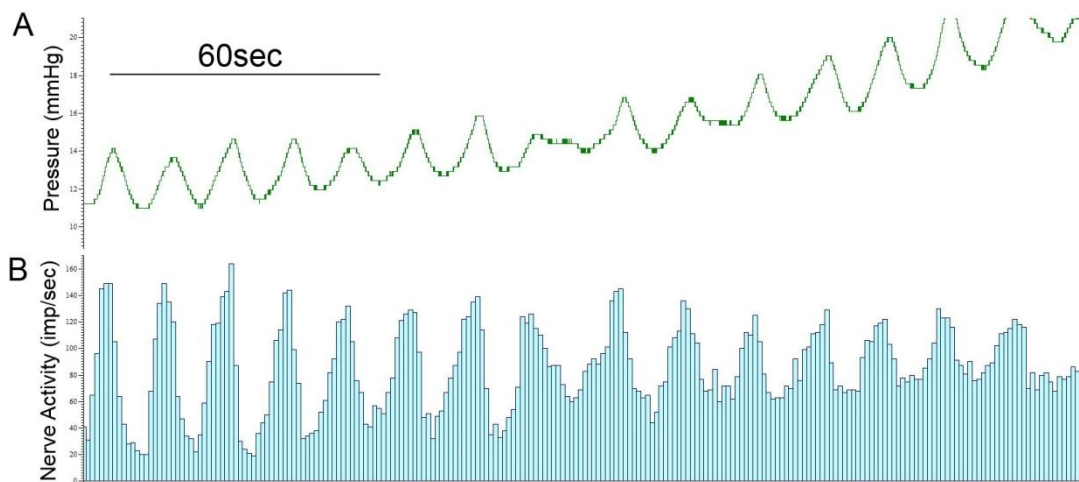


Figure 5.5: Representative traces of a control isolated mouse bladder showing spontaneous phasic contractions (A) during filling and the associated nerve activity (B).

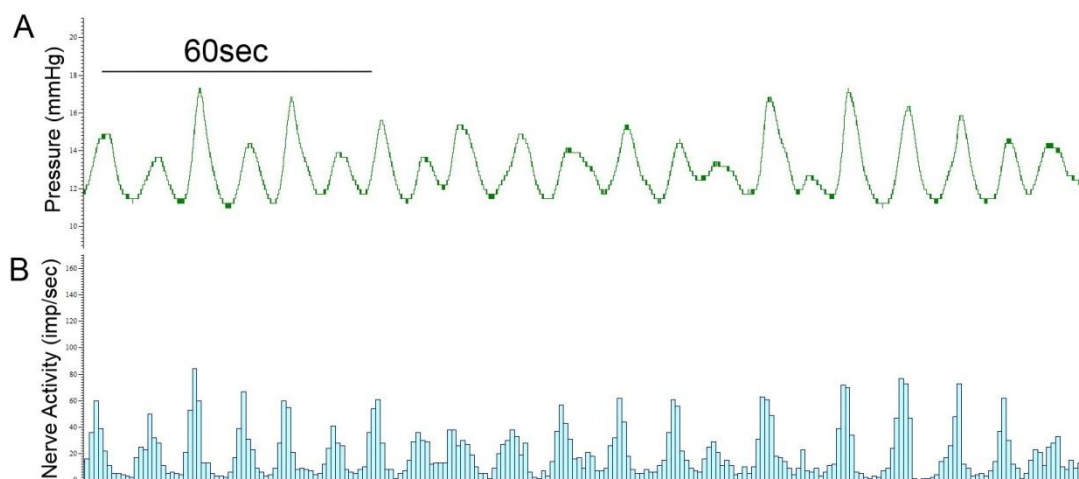


Figure 5.6: Representative traces of a control isolated mouse bladder showing spontaneous phasic contractions (A) during accommodation and the associated nerve activity (B).

Response to Pharmacological Agents

To study the effect of a drug on afferent response or muscle tension, the bladder was filled to a set pressure of 20mmHg and allowed to stabilise for an hour. The muscle then had a stable baseline tension and afferent output from which to determine the effect of the drug. Agonists were then added to the bath and the effect of the drug on afferent activity was calculated by measuring the mean peak response (imp/sec) and subtracting the mean baseline activity 30 seconds before drug application. Bladder contractions were determined as the changes in intravesical pressure from baseline pressure and expressed in mmHg.

Drugs were also applied to the luminal surface of the bladder through the urethral catheter. Drugs were diluted in isotonic saline to the desired concentration and instilled through the urethral catheter via the syringe driver. With the out-flow tap open, the drug was infused at 30 μ l/min so there was no change to intravesical pressure. Afferent activity was monitored during drug infusion as well as during distensions with the drug solution.

Single Unit and Principal Component Analysis

Single-unit (single nerve fibre) discrimination was performed offline using the Spike2 wavemark analysis function which discriminates between the distinct action potential characteristics of individual nerve fibres including waveform, amplitude and duration. The action potentials were analysed and templates were constructed based on their distinct shape in order to identify the individual nerve fibres in each preparation (**Figure 5.7**).

Afferent activity was sampled at 20,000 Hz such that a 1.8msec period consisting of approximately 28 data points was used to construct each template. Templates were used to allocate every action potential throughout a recording to a distinct single unit as shown by the wavemark trace in (**Figure 5.7-C**). The parameters for a spike to match a template were typically <10% variability in amplitude and >60% of data points within the template boundary.

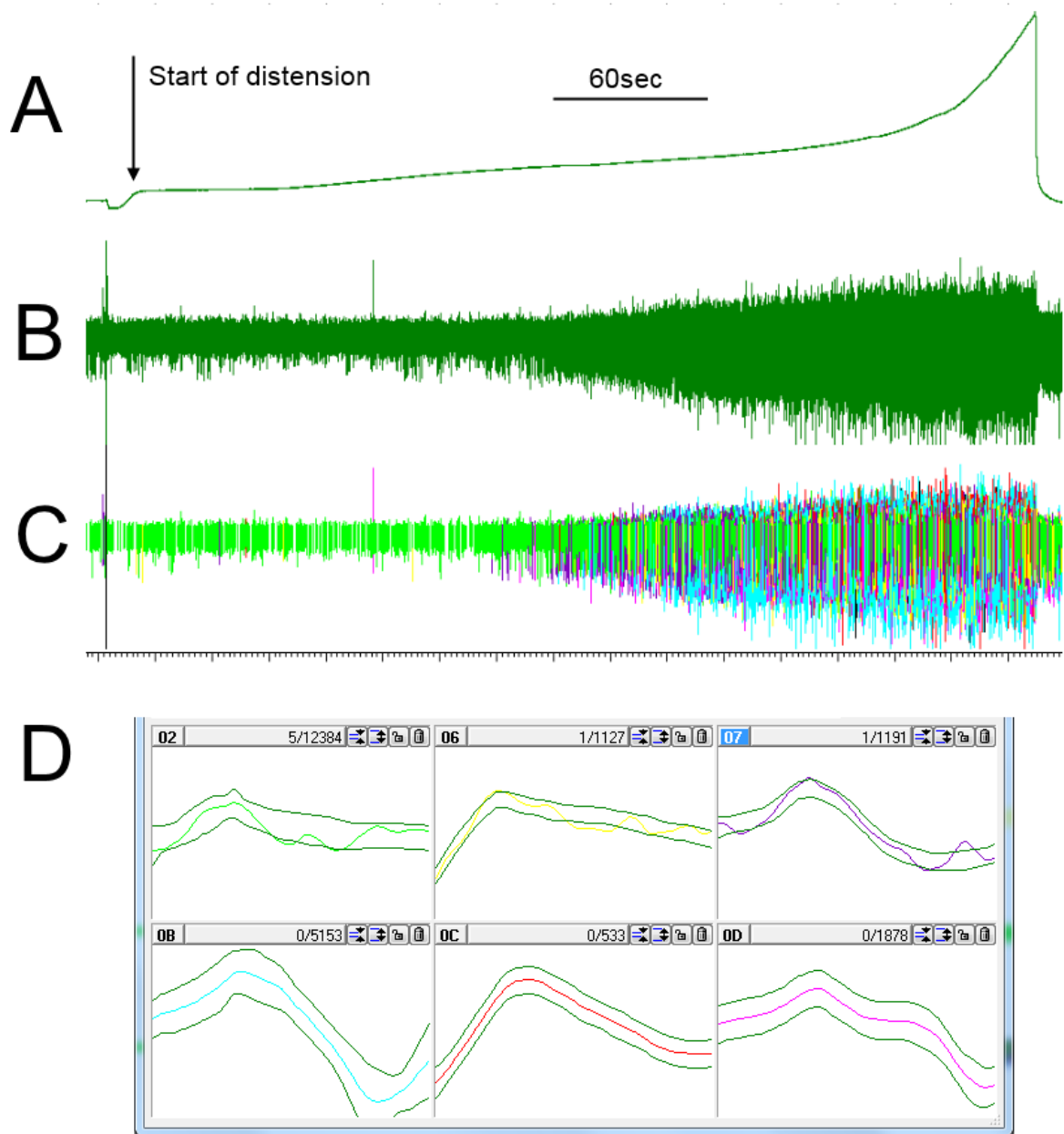


Figure 5.7: Characterisation of the single nerve fibres active during distension of a mouse bladder (A) wherein the total nerve activity (B) is differentiated into six distinct fibres (C) based on action potential shape (D).

Principal component analysis (PCA) was then used to ensure that the spike templates identified were different enough to be classified as distinct nerve fibres. PCA uses algorithms to extract features from the data that contribute most to differences between spike shapes such as amplitude, latency, area or slope (**Figure 5.8-A**). PCA scales and represents the differences between spike shapes in 3-dimensions so that spikes having a similar shape are clustered together. **Figure 5.8-B** is a 2-dimensional representation of this display where each coloured cluster represents spikes matching a single template. The amount of overlap between the clusters was evaluated by eye and clusters which had significant overlap were considered too similar to be from distinct nerves and were classified as originating from one nerve fibre.

As well as total nerve activity, fibre averaging analysis was also performed in the following experiments. Fibre averaging analysis involves determining the activity of each individual fibre in a bundle and averaging them so that the impulses per second per fibre can be determined for each preparation. This reduces the variation in results due to differences in the number of fibres in a nerve bundle. Using templates and PCA, the number of distinct fibres isolated in each experiment was determined and the nerve activity was then averaged to give the nerve activity per single fibre.

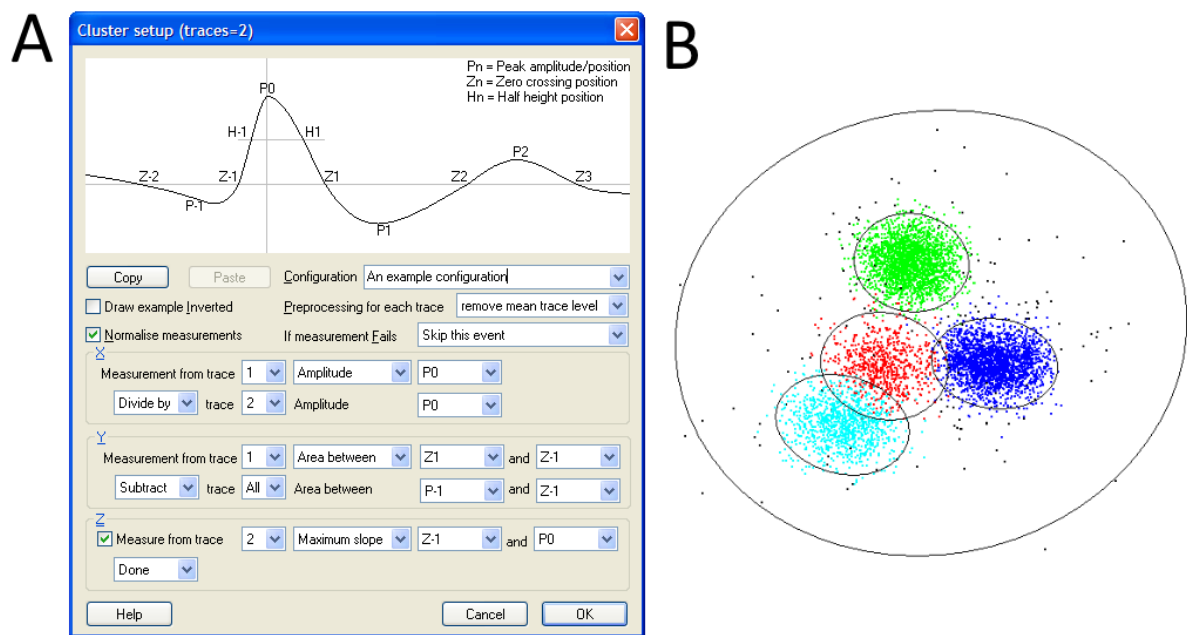


Figure 5.8: A screen shot showing the characteristics used to cluster action potentials allocated to each template (A) and a 2-dimensional representation of the 3-dimensional clustering of action potentials according to the characteristics selected, with 4 distinct single unit clusters shown (B).

Single Unit Classification

Once the distinct single units of each bundle were identified, the fibres were then classified based on the pressure at which they were activated. Two broad classifications were used, low threshold fibres and high threshold fibres. Low threshold fibres were defined as those fibres responding at pressures below 15mmHg and high threshold as those responding at pressures above 15mmHg. Classifying nerves as high or low threshold using 15mmHg is consistent with previous studies (Daly et al., 2007, Rong et al., 2002) and is approximately the micturition threshold in the mouse (Igawa et al., 2004). **Figure 5.9** shows a recording from a nerve bundle containing three distinct fibres, one of which responds only to pressures above 15mmHg (D) and would be classified as a high threshold fibre, while the other two fibres (E & F) show activity at pressures below 15mmHg and would be classified as low threshold fibres.

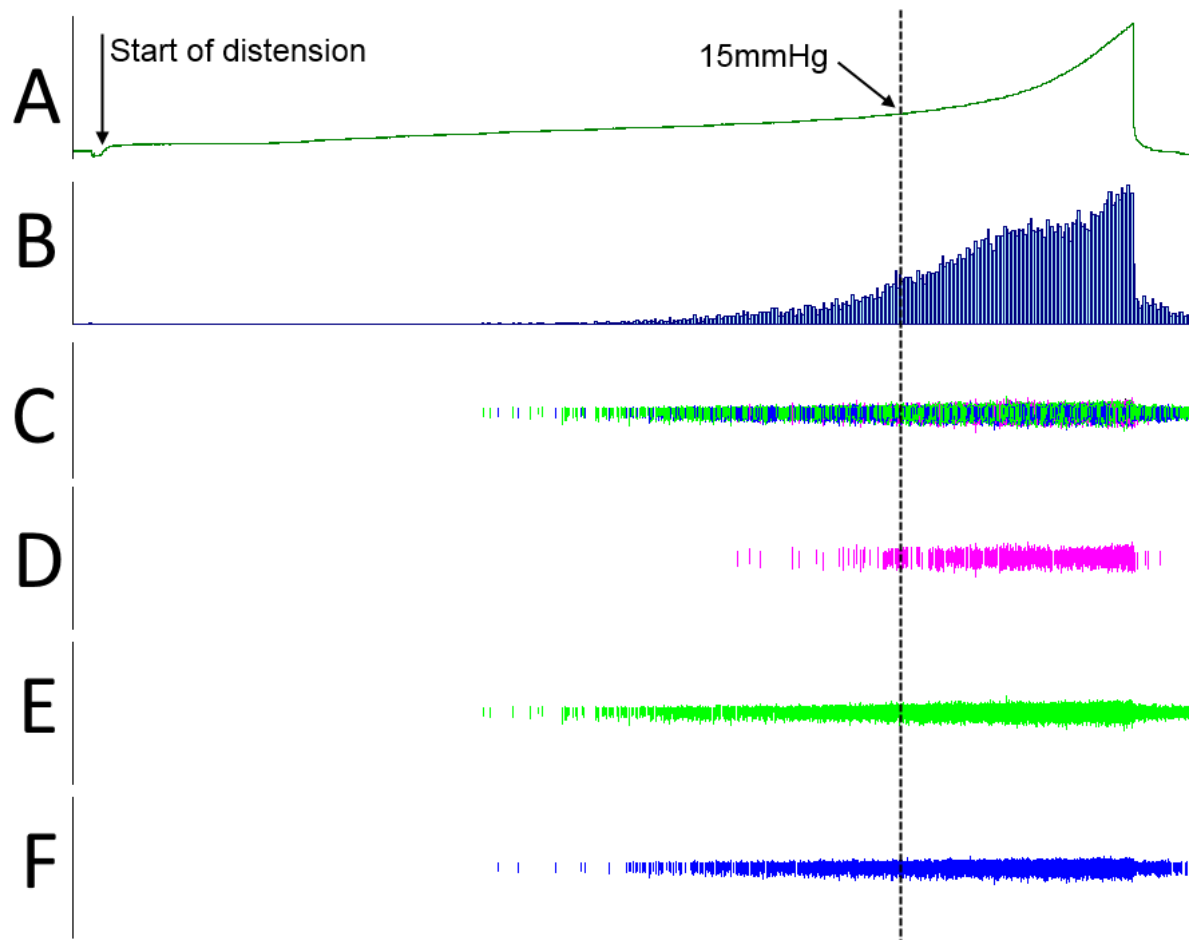


Figure 5.9: Representative trace during distension of a mouse bladder to 40mmHg (A), showing total nerve activity (B) characterised into three distinct nerve fibres (C) and the activity of each fibre isolated and classified as one high threshold fibre (D) and two low threshold fibres (E & F).

Experimental Protocol

At the beginning of each experiment, the viability of the preparation was checked. If the nerve failed to give typical reproducible responses to distension the nerve was removed from the electrode and another nerve branch was selected. The nerve preparation was left to stabilise for 30-60 minutes with the bladder empty. Then, a control distension was performed by closing the outflow tap, filling the bladder with saline (0.9% NaCl) at 30 μ l/min to a pressure of 40mmHg and opening outflow tap allowing the bladder to empty. This was repeated every 10 minutes until a stable pressure and afferent response to distension was observed (see **Figure 5.10**).

The bladder was then distended at 30 μ l/min to 20mmHg and left with the outflow tap closed for 1 hour to allow accommodation of the volume and stabilisation of bladder tone and nerve activity. After 1 hour the internal pressure and the afferent output had stabilised (**Figure 5.11**).

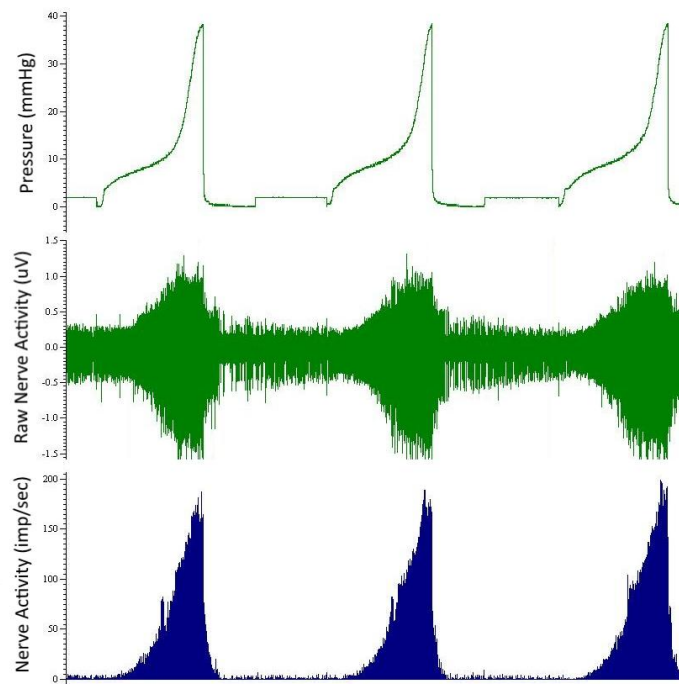


Figure 5.10: Representative traces showing repeatable intravesical pressure (mmHg), raw nerve activity (μ V) and nerve activity (imp/sec) during three distensions of a mouse bladder.

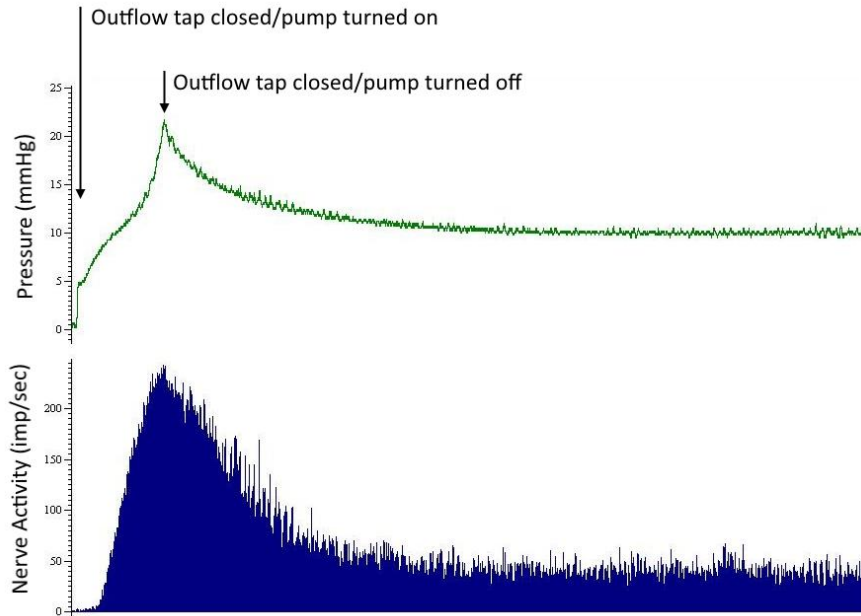


Figure 5.11: Representative trace of a mouse bladder showing stabilisation of intravesical pressure (mmHg) and nerve activity (imp/sec) during accommodation of a volume.

After the detrusor tone and afferent activity stabilised, a variety of pharmacological agents were then sequentially added to the bath as per the following list, allowing at least 30 minutes superfusion to wash the tissue between each drug addition:

- ➔ ATP (1mM) (purinoceptor agonist)
- ➔ $\alpha\beta$ methylene ATP (10 μ M) (P2X purinoceptor agonist)
- ➔ ACh (1 μ M) (muscarinic and nicotinic receptor agonist)
- ➔ Carbachol (10 μ M) (muscarinic receptor agonist)
- ➔ Polygodial (10 μ M) (TRPA1 channel activator)
- ➔ DMPP (10 μ M) (nicotinic receptor agonist)
- ➔ WS-12 (10 μ M) (TRPM8 channel activator)
- ➔ PGE₂ (1 μ M) (E-prostanoid receptor agonist)
- ➔ SNP (1mM) (NO donor)
- ➔ Capsaicin (10 μ M) (TRPV1 channel activator)

In separate experiments the responses to ATP (1 μ M), $\alpha\beta$ methylene ATP (10 μ M), carbachol (10 μ M) and DMPP (10 μ M) were repeated in the absence and presence of nifedipine (1 μ M) an L-type calcium channel blocker that blocks smooth muscle contraction and associated indirect nerve responses, thereby uncovering the direct nerve responses to the drug alone. These experiments were performed in control and CPO (100mg/kg) treated mice only. Firstly, the drugs were sequentially added in the absence of nifedipine with appropriate wash times

between each drug addition. Next, Krebs solution containing nifedipine (1 μ M) was superfused continuously over the tissue for 30 minutes prior to adding the drugs again in the same sequence with the same wash periods between each drug addition. The pressure and nerve responses remaining in the presence of nifedipine (1 μ M) were represented as a percentage of the response in the absence of nifedipine. Responses in CPO treated bladders were compared to responses in control bladders.

Statistical Analysis

Results were expressed as mean \pm standard error of the mean (SEM). Data were analysed using a Student t-test or one-way ANOVA with Dunnett or Tukey multiple comparisons test as appropriate, using Graphpad InStat (version 3.06) software (SanDiego, CA). Comparisons of whole response curves of control and treated groups were performed using two-way ANOVA with Bonferroni post-test. Significance levels were defined as $p < 0.05$ (*), $p < 0.01$ (**) and $p < 0.001$ (***)).

5.3 RESULTS

Before treatment, there was no significant difference in age or weight between animals in each treatment group while CPO (100mg/kg) and IFO (200mg/kg) treatment caused greater weight loss after 24 hours than in control animals ($p<0.01$). Average bladder volume was not different between the three groups ($347\pm31\mu\text{l}$ in controls, $272\pm35\mu\text{l}$ in CPO treated and $365\pm54\mu\text{l}$ in IFO treated animals).

Intravesical Pressure and Nerve Activity During Bladder Distension

Firstly, the effects of CPO and IFO on bladder pressure and nerve activity during graded distensions were investigated. As volume in the bladder increased both intravesical pressure and bladder sensory nerve activity increased. Both the muscle and nerve of each mouse bladder demonstrated stable and repeatable responses during distensions at $30\mu\text{l}/\text{min}$ to 40mmHg . The afferent nerve response was almost completely abolished by tetrodotoxin (TTx) ($1\mu\text{M}$) (Figure 5.12).

Bladder filling typically included an initial gentle increase in tension to approximately 15mmHg accompanied by a large increase in afferent nerve activity. This phase was followed by a steep increase in tension accompanied by a smaller increase in afferent nerve activity. This pattern of filling occurred in all bladders in all treatment groups despite variations in total bladder volume and volumes accommodated in each phase.

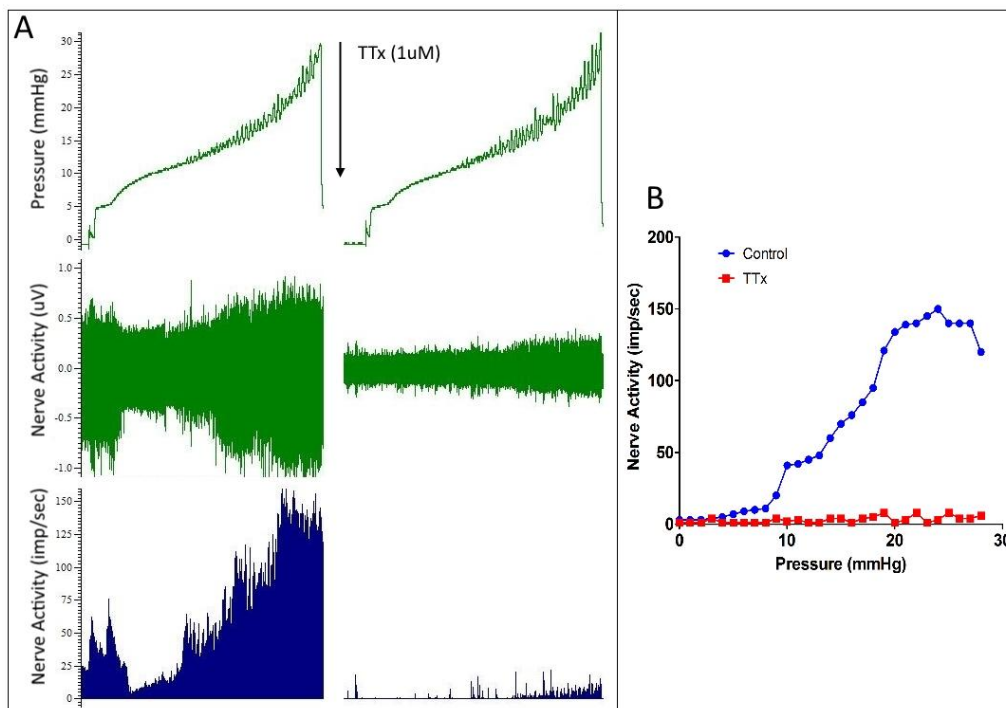


Figure 5.12: Representative trace (A) and graph (B) showing the effect of tetrodotoxin (TTx) ($1\mu\text{M}$) on the afferent nerve activity during distension of a mouse bladder ($n=1$).

Initially, IFO was examined at an equal dose to CPO (100mg/kg) to investigate the contribution of CAA to any changes in response. However, at this lower dose, IFO (100mg/kg) had no effect on compliance of the muscle (**Figure 5.13-A**) or afferent nerve activity (**Figure 5.13-B**) during distensions (n=4) and the more clinically relevant dose of 200mg/kg was used for further experiments.

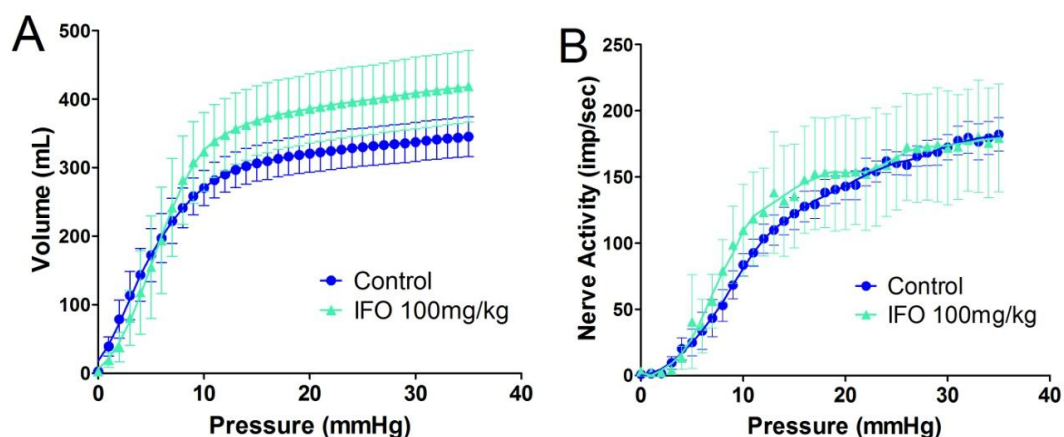


Figure 5.13: Compliance (A) and afferent nerve activity (B) of mouse bladders treated with saline (control) or IFO (100mg/ml). Data represented as mean \pm SEM (n \geq 4). Analysed by two-way ANOVA.

Treatment with CPO (100mg/kg) or IFO (200mg/kg) had no effect on the compliance of the bladder muscle during distensions (**Figure 5.14-A**). However, nerve activity after treatment with either CPO or IFO was enhanced throughout bladder filling (**Figure 5.14-B**). At maximum distension the total nerve activity was increased significantly from 182 ± 13 impulses per second (imp/sec) in control animals, to 230 ± 14 imp/sec in CPO treated mice ($p < 0.05$) and 226 ± 17 imp/sec in IFO treated mice ($p < 0.001$) (n \geq 6). However, the difference was even greater at 15mmHg where CPO and IFO increased nerve activity from 122 ± 12 imp/sec in controls to 189 ± 17 imp/sec and 180 ± 22 imp/sec respectively.

The single units were identified in each preparation and the average number of distinct nerve fibres was not significantly different between treatment groups. Nerve bundles from all groups contained between 6-13 distinct nerve fibres with the average being 8.8 ± 0.8 fibres in control, 8.1 ± 0.6 fibres in CPO and 9.2 ± 0.5 fibres in IFO treated animals (**Figure 5.15**). These single fibres were also classified based on their activation threshold and the number of both low and high threshold fibres was similar between groups. Low threshold fibres were more numerous than high threshold fibres in all treatment groups ranging between 4-10 low threshold fibres compared to 0-4 high threshold fibres in each bundle. Accordingly, low threshold fibres made up $59.8 \pm 8.8\%$ of the total fibre population in control bladders and while not significant, treatment with CPO or IFO showed a trend towards an increasing population of low threshold fibres to $76.5 \pm 7.1\%$ and $75.8 \pm 6.3\%$ respectively (**Figure 5.16**).

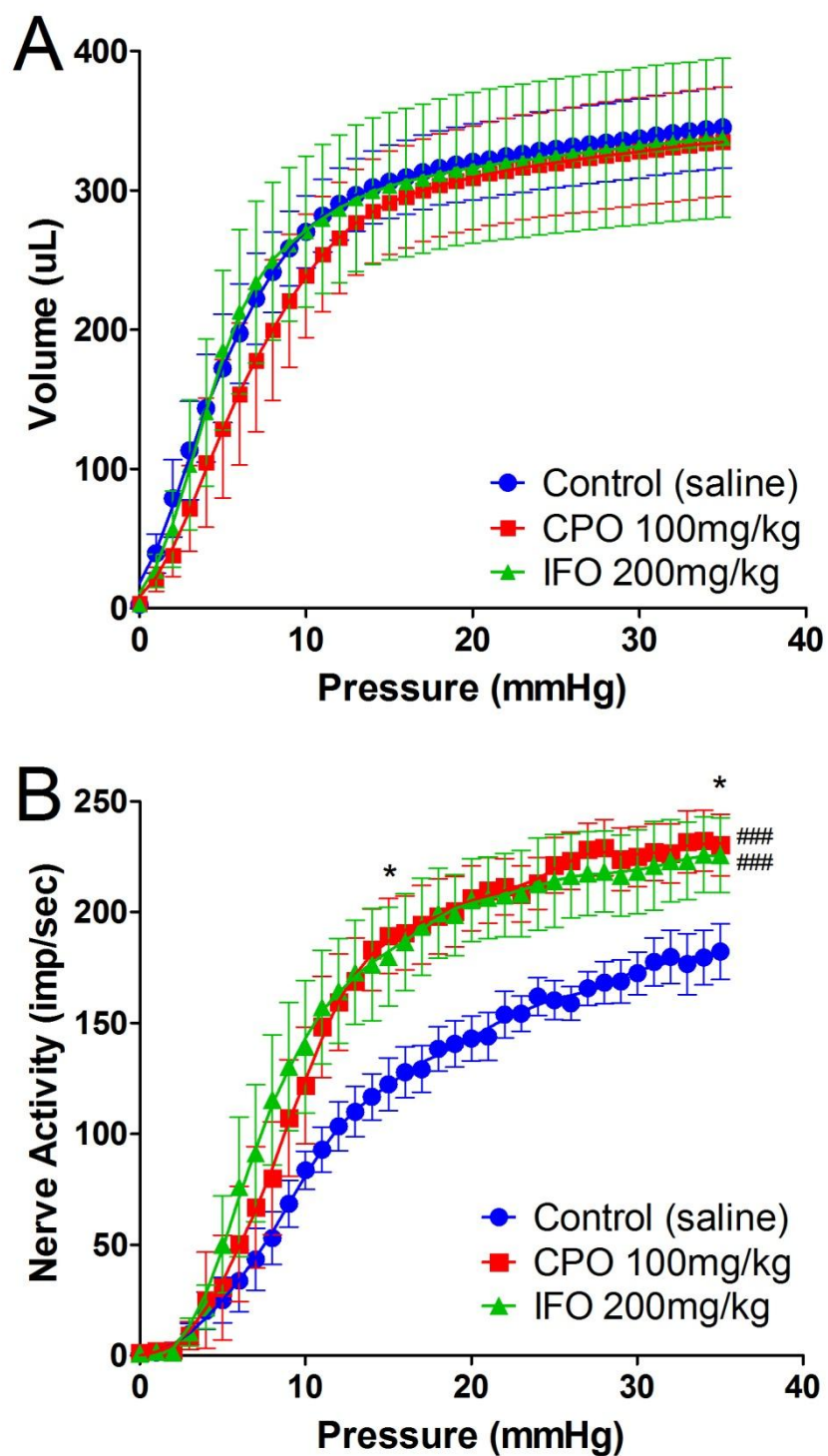


Figure 5.14: Compliance (A) and afferent nerve activity (B) of mouse bladders treated with saline (control), CPO (100mg/ml) or IFO (200mg/ml). Data represented as mean \pm SEM ($n \geq 6$). Analysed by two-way ANOVA (* $p < 0.05$ vs comparable control activity at individual pressure interval, ### $p < 0.001$ vs whole control curve).

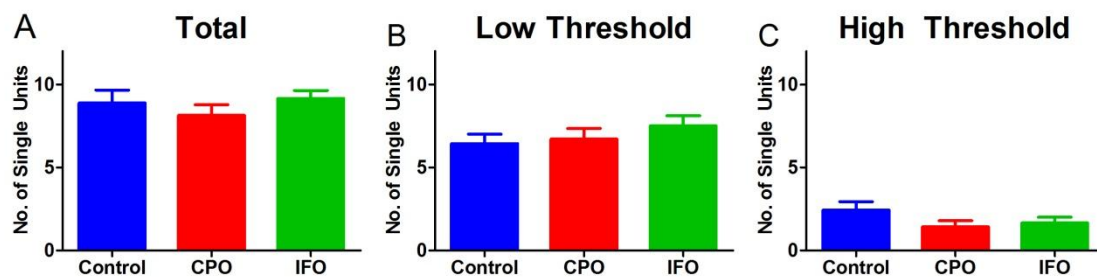


Figure 5.15: The average number of individual nerve fibres (single units) identified in each mouse bladder preparation (A) as well as the number of these fibres classified as low threshold fibres (B) and high threshold fibres (C). Data represented as mean \pm SEM (n \geq 6).

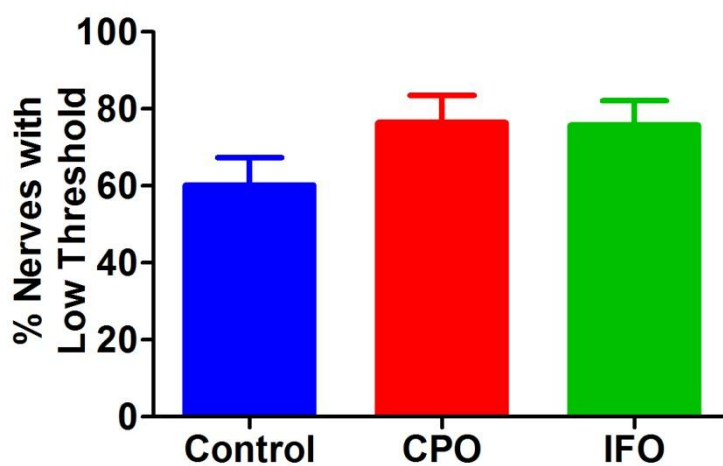


Figure 5.16: The percentage of nerves classified as low threshold in saline (control), CPO (100mg/kg) or IFO (200mg/kg) treated mice. Data represented as mean \pm SEM (n \geq 6).

The activity of each distinct nerve fibre was determined and fibre averaging analysis performed to obtain the average activity per fibre in each preparation. At maximum distension the average activity per fibre was significantly increased from 19.1 ± 1.1 impulses per second per fibre (imp/sec/fibre) in control animals, to 29.1 ± 1.8 imp/sec/fibre in CPO treated mice ($p < 0.01$) and 24.9 ± 1.4 imp/sec/fibre in IFO treated mice ($p < 0.05$) (**Figure 5.17**).

The effect of CPO or IFO treatment on the activity of low and high threshold fibres was assessed next. As intravesical pressure increased, low threshold fibres demonstrated a rapid increase in nerve activity up to approximately 15mmHg at which point further increases in activity were gradual and activity started to plateau at maximum distension (**Figure 5.18-A**). High threshold fibres became active when intravesical pressure reached approximately 15mmHg and then continued to increase in activity at a steady rate to maximum distension (**Figure 5.18-A**). Low threshold fibres had a greater firing rate at maximum distension (23.3 ± 1.9 imp/sec/fibre) compared to high threshold fibres (14.5 ± 1.7 imp/sec/fibre) ($p < 0.01$).

The activity of high threshold nerves was unchanged after treatment with cyclophosphamide or ifosfamide (**Figure 5.18-C**), whereas treatment caused enhanced activity of the low threshold nerves ($p < 0.05$) (**Figure 5.18-B**). Low threshold nerve activity at maximum distension increased significantly from 23.3 ± 1.9 imp/sec/fibre in control animals, to 31.5 ± 2.5 imp/sec/fibre in CPO treated mice ($p < 0.01$) and 29.9 ± 2.0 imp/sec/fibre in IFO treated mice ($p < 0.05$) ($n \geq 44$).

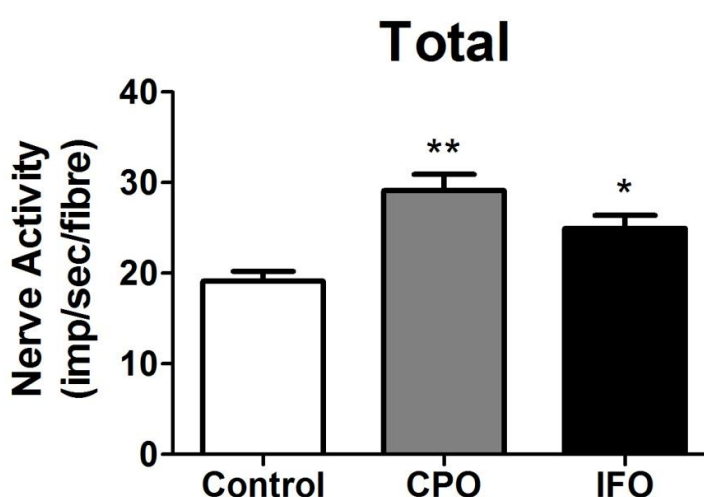


Figure 5.17: Average maximum nerve activity (imp/sec) per individual nerve fibre during bladder distension at 40mmHg in bladders from saline (control), CPO (100mg/kg) or IFO (200mg/kg) treated mice. Data represented as mean \pm SEM ($n \geq 6$). Analysed by one-way ANOVA with Dunnett post test (* $p < 0.05$, ** $p < 0.01$ vs control).

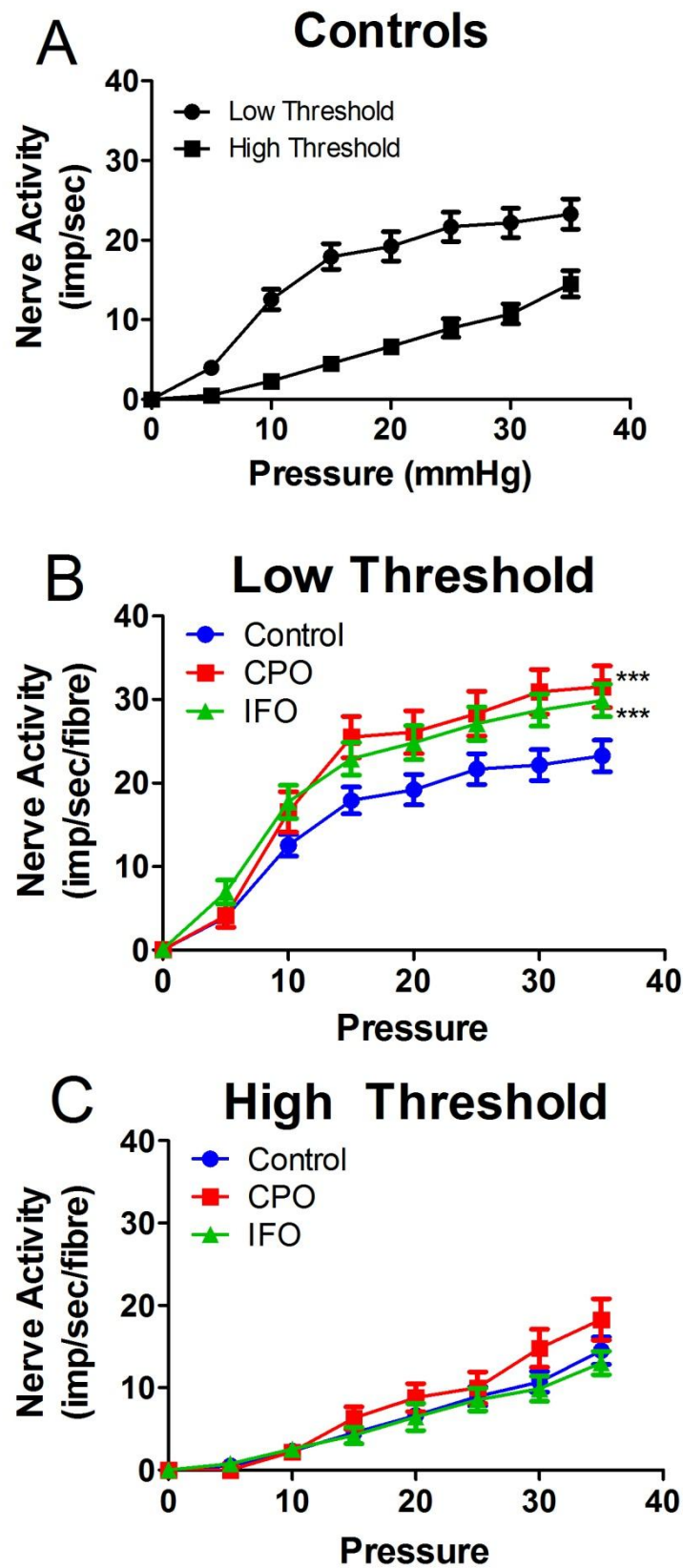


Figure 5.18: Afferent nerve activity of low (n=52) and high threshold fibres (n=22) in control animals (A) and the effect of treatment with CPO (100mg/ml) or IFO (200mg/ml) on low threshold nerve activity (B) (n≥44) and high threshold nerve activity (C) (n≥10). Data represented as mean ± SEM and analysed by two-way ANOVA (**p<0.001 vs control curve).

In low threshold fibres, two response patterns were observed. In the first pattern, low threshold afferents continued to encode up to maximum distension (**Figure 5.19-A**). The second group demonstrated a non-encoding pattern wherein firing rate underwent an initial increase and then began to plateau or even decreased in activity from approximately 15mmHg (**Figure 5.19-B**). The non-encoding fibres were more numerous than the encoding fibres in control animals making up 67% of the low threshold fibres. This percentage was slightly less in CPO and IFO treated animals with non-encoding fibres making up 58% and 52% of all low threshold fibres respectively although the difference was not significant. At maximum distension, treatment with CPO or IFO significantly enhanced the activity of the encoding fibres from 22.1 ± 2.7 imp/sec/fibre to 37.8 ± 3.1 imp/sec/fibre and 35.4 ± 3.7 imp/sec/fibre respectively ($p < 0.05$). Treatment did not affect the activity of the non-encoding fibres.

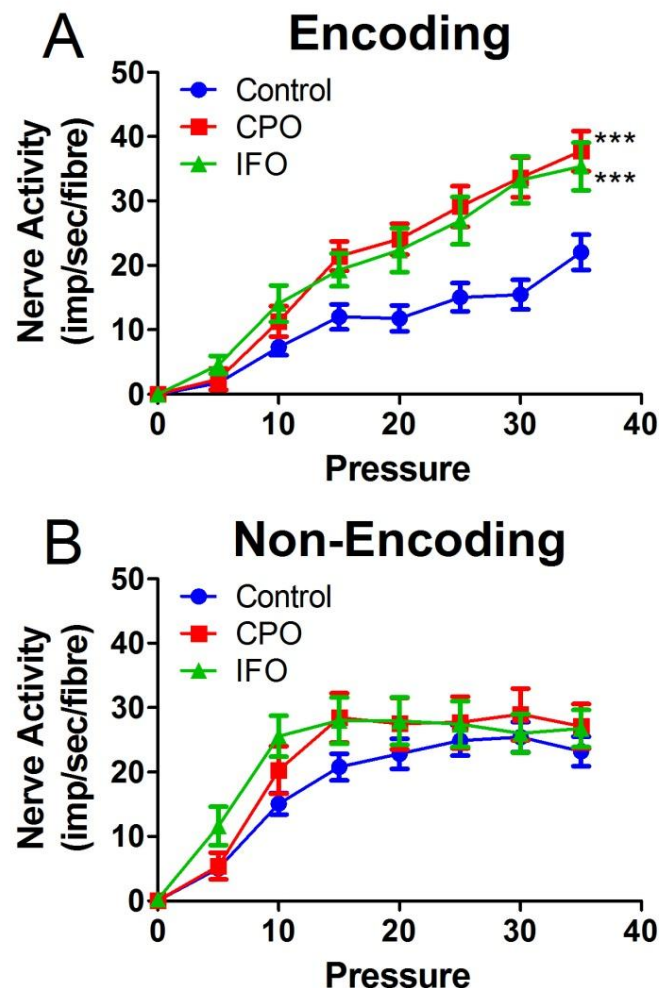


Figure 5.19: Afferent nerve activity in low threshold fibres divided into encoding (A) ($n \geq 17$) and non-encoding (B) firing patterns ($n \geq 18$) in control animals, CPO (100mg/ml) or IFO (200mg/ml) treated animals. Data represented as mean \pm SEM and analysed by two-way ANOVA (** $p < 0.001$ vs control curve).

Spontaneous Contractions

Given that increased spontaneous contractions of the bladder can be associated with dysfunction, the effect of CPO and IFO on the frequency and amplitude of phasic activity during filling and accommodation were measured. Treatment with CPO (100mg/kg) or IFO (200mg/kg) had no significant effect on the average frequency (**Figure 5.20-A**) or the amplitude (**Figure 5.20-B**) of spontaneous contractions compared to control. The nerve activity showed phasic increases associated with the muscle contractions and the phasic nerve activity was the same in control and CPO treated tissues (**Figure 5.20-C**). Interestingly, the spontaneous detrusor contractions persisted in the presence of tetrodotoxin (1 μ M) which abolished the nerve activity (**Figure 5.21**).

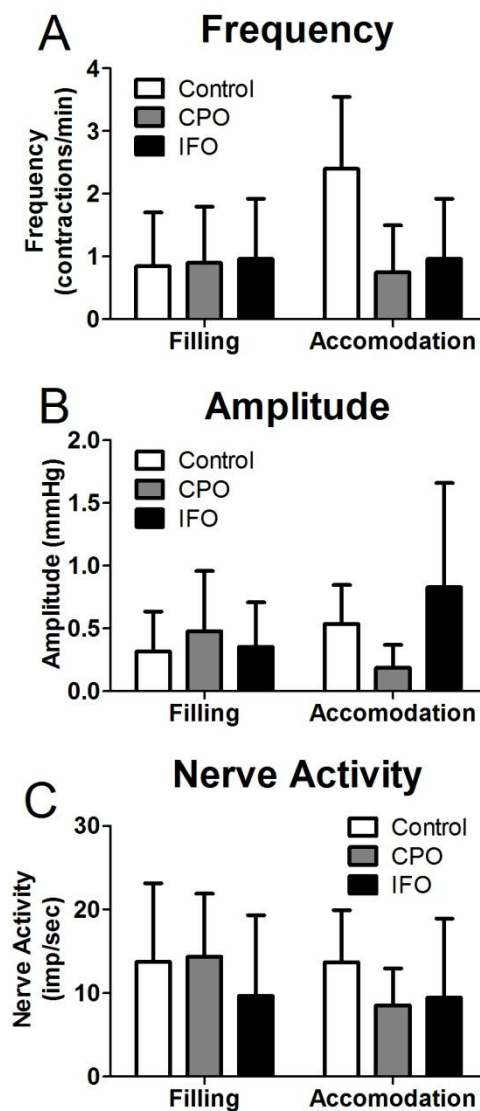


Figure 5.20: Average frequency (A) and amplitude (B) of spontaneous contractions in bladders from saline (control), CPO (100mg/kg) and IFO (200mg/kg) treated mice during filling and accommodation. Data represented as mean \pm SEM (n \geq 4). Analysed by one-way ANOVA with Tukey post-test.

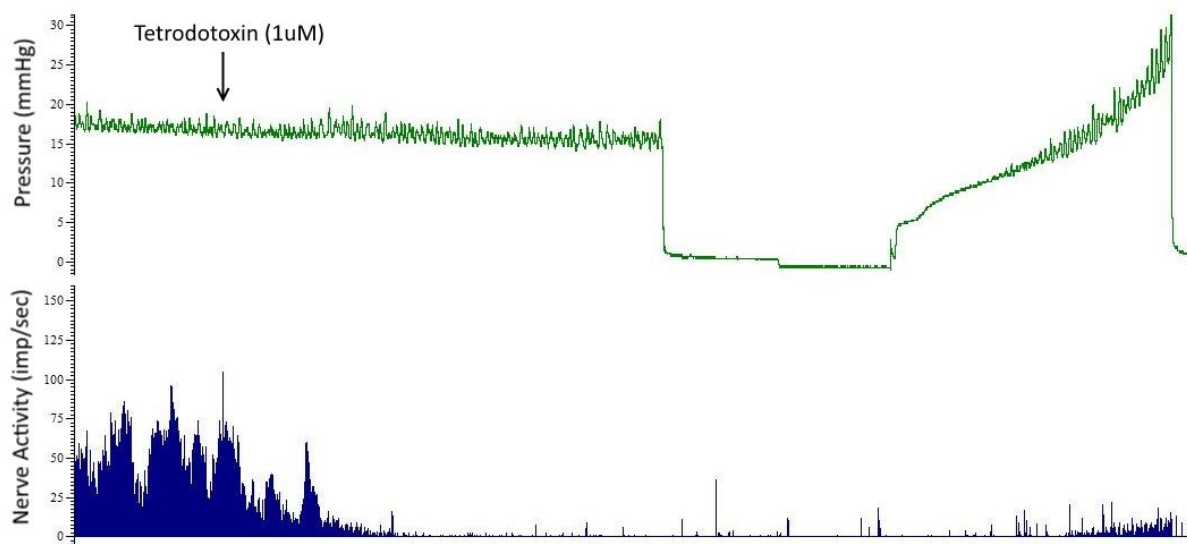


Figure 5.21: Representative trace showing the detrusor contractions and nerve activity in a bladder pre-distended to 20mmHg before and after tetrodotoxin (1 μ M) followed by a normal distension.

Response to Pharmacological Agents

The bladder muscle and afferent nerves can respond to various pharmacological agents and changes in these responses may contribute to atypical bladder function. Initially the bladder was filled to 20mmHg and left until pressure and nerve activity were stable in order to quantify the responses to various agonists. Compliance of the bladder muscle during accommodation from 20mmHg intravesical pressure was unchanged by treatment with CPO (100mg/kg) or IFO (200mg/kg) (**Figure 5.22-A**). The total nerve activity was increased at 20mmHg in both CPO and IFO treated animals, however the nerve activity was able to adapt over time to be similar to the activity in control animals (**Figure 5.22-B**). Control, CPO and IFO treated bladders stabilised to a similar intravesical pressures (6.01 ± 0.71 mmHg, 6.96 ± 0.65 mmHg and 7.00 ± 1.62 mmHg respectively) and nerve firing rates (32.0 ± 16.1 imp/sec, 43.5 ± 23.2 imp/sec and 24.2 ± 7.62 imp/sec respectively) after approximately 30 minutes. The effect of pharmacological agents was then determined from this stable tension and afferent activity.

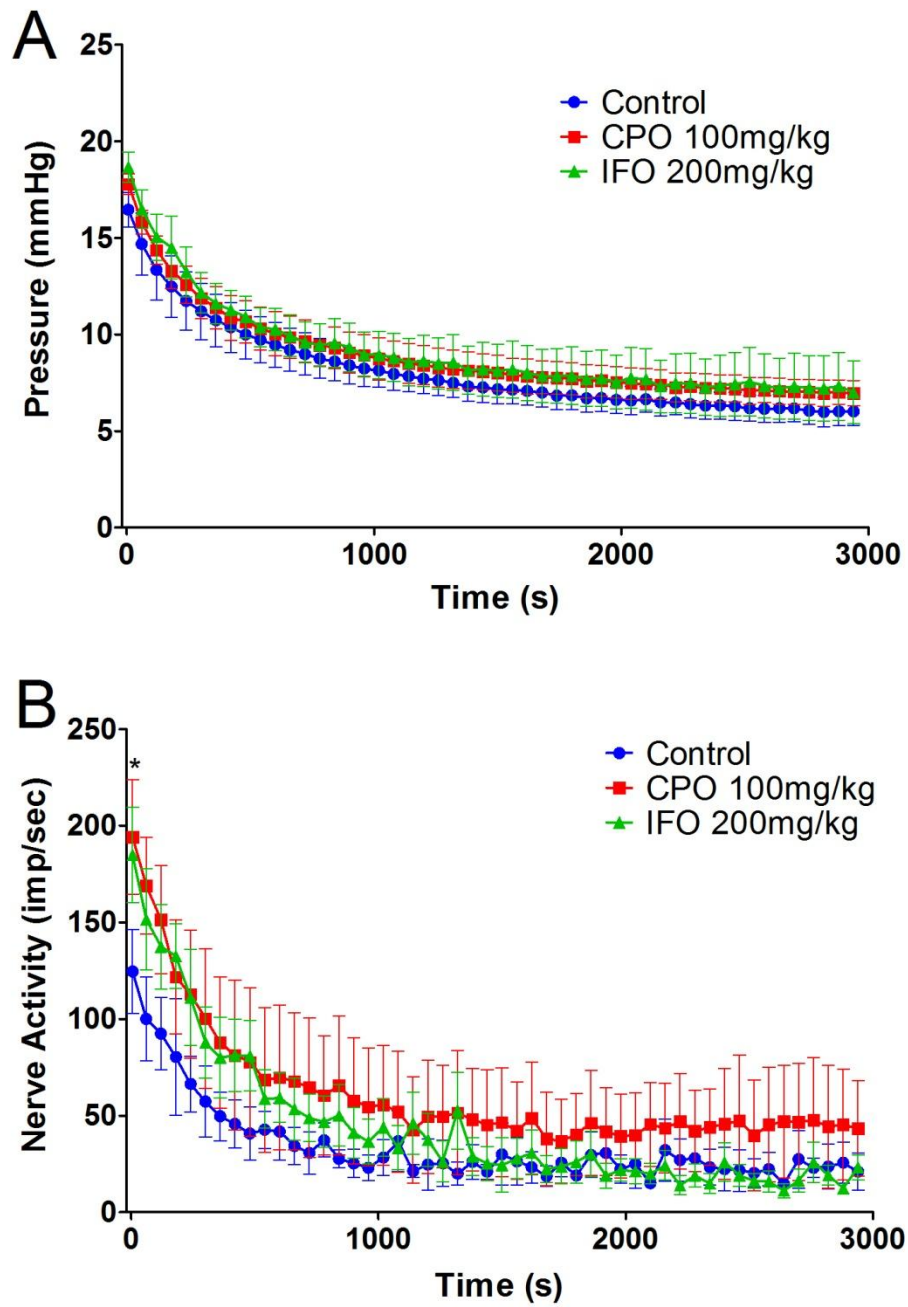


Figure 5.22: Compliance (A) and afferent nerve activity (B) during accommodation from 20mmHg intravesical pressure in mouse bladders treated with saline (control), CPO (100mg/ml) or IFO (200mg/ml). Data represented as mean \pm SEM (n=5). First and final time point analysed by one-way ANOVA with a Dunnett post-test (* $p < 0.05$).

In control preparations, extraluminal application of ATP (1mM) caused a contraction of the bladder smooth muscle and an associated increase in intravesical pressure (**Figure 5.23**) which returned back to baseline tension within 10 minutes. This was accompanied by a simultaneous increase in total afferent activity and recovery to baseline firing. The addition of $\alpha\beta$ mATP (10 μ M) resulted in a larger increase in intravesical pressure and total afferent activity which also recovered to baseline levels within 10 minutes.

A contraction was also observed after adding ACh (1 μ M) and the muscle developed phasic contractions (**Figure 5.23**). Recovery to baseline pressure took up to 30 minutes by which time the phasic contractions had returned to baseline levels. The changes in pressure were also associated with increases in total nerve activity and recovery to pre-drug firing over 30 minutes. Carbachol (10 μ M) induced a larger increase in nerve activity and intravesical pressure compared to ACh and took up to one hour to recover to baseline levels. Phasic contractions seen after carbachol (10 μ M) were also larger and more frequent than after ACh (1 μ M).

Activation of nicotinic receptors by DMPP (10 μ M) caused an increase in nerve activity while having very little or no effect on intravesical pressure (**Figure 5.23**). Application of PGE₂ (1 μ M) typically caused a small but long lasting increase in intravesical pressure. Baseline nerve activity after PGE₂ application did not change, however spontaneous bursts of activity appeared. Capsaicin (10 μ M) caused a small and brief intravesical pressure increase. Nerve activity in response to capsaicin also increased briefly, then decreased to a level below the baseline firing rate and eventually recovered back to baseline after a long washout period of at least 30 minutes. Typically no pressure or nerve responses were seen after application of polygodial (10 μ M), WS-12 (10 μ M) or SNP (1mM).

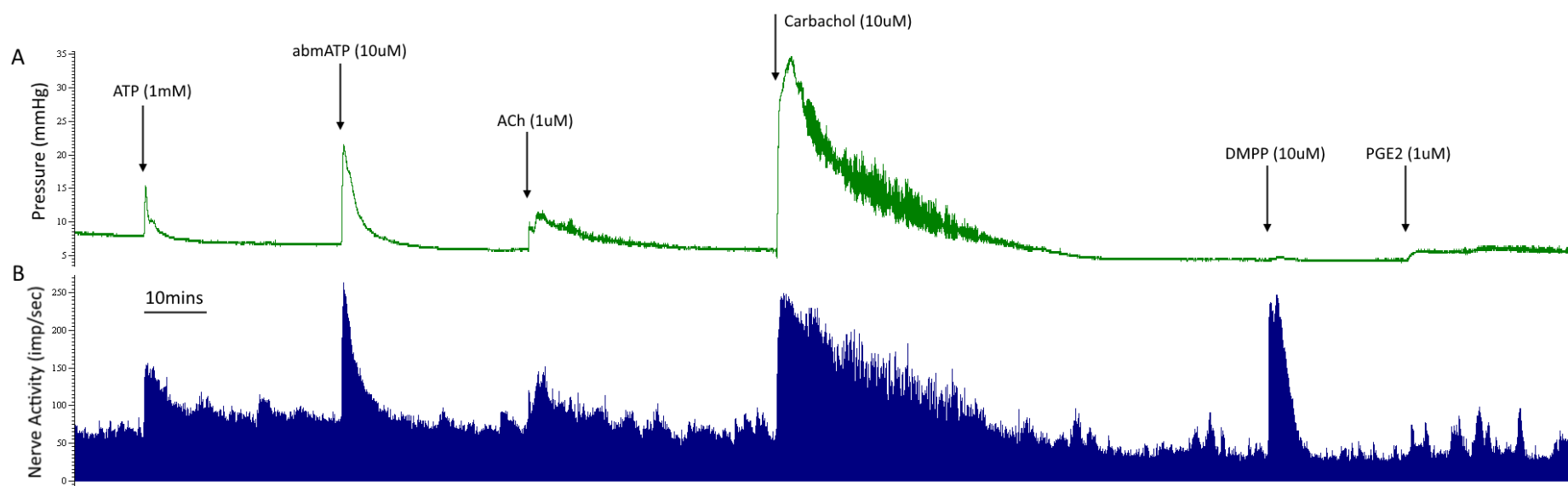


Figure 5.23: Representative trace of intravesical pressure (A) and nerve activity (B) in a mouse bladder in response to extraluminal addition of ATP (1mM), $\alpha\beta$ mATP (10 μ M), ACh (1 μ M), carbachol (10 μ M), DMPP (10 μ M) and PGE₂ (1 μ M).

Compared to controls, the pressure responses to $\alpha\beta$ mATP (10 μ M) were not changed after CPO or IFO treatment and while responses to ATP (1mM) were not significantly different there was a slight decrease after treatment (**Figure 5.24-A**). Nerve responses to $\alpha\beta$ mATP (10 μ M) in CPO or IFO treated bladders were also unchanged compared to controls and responses to ATP (1mM) were also not significantly different despite showing a decreasing trend (**Figure 5.24-B**).

Muscle responses to ACh (1 μ M) were increased by 30% in CPO treated bladders and reduced by 40% in IFO treated bladders, however this was not significant. Nerve activity responses to ACh (1 μ M) mirrored these effects and were slightly increased after CPO treatment (40%) and reduced after IFO treatment (30%). Pressure and nerve responses to carbachol (10 μ M) were also slightly increased in both CPO and IFO treated groups (10-20%) but not significantly.

Bladders treated with CPO or IFO also demonstrated a trend towards increased nerve activity in response to DMPP (10 μ M) and PGE₂ with effects appearing greater after IFO treatment. Finally, in some of the CPO or IFO treated bladders, administration of SNP (1mM) increased the frequency of the bursts of nerve activity induced by PGE₂ and increased the rate of firing during those bursts, while producing a very slight increase in intravesical pressure. Overall, changes in response to pharmaceutical agents were greater in IFO treated bladders than CPO treated however these effects were only significant for the IFO induced nerve response to SNP.

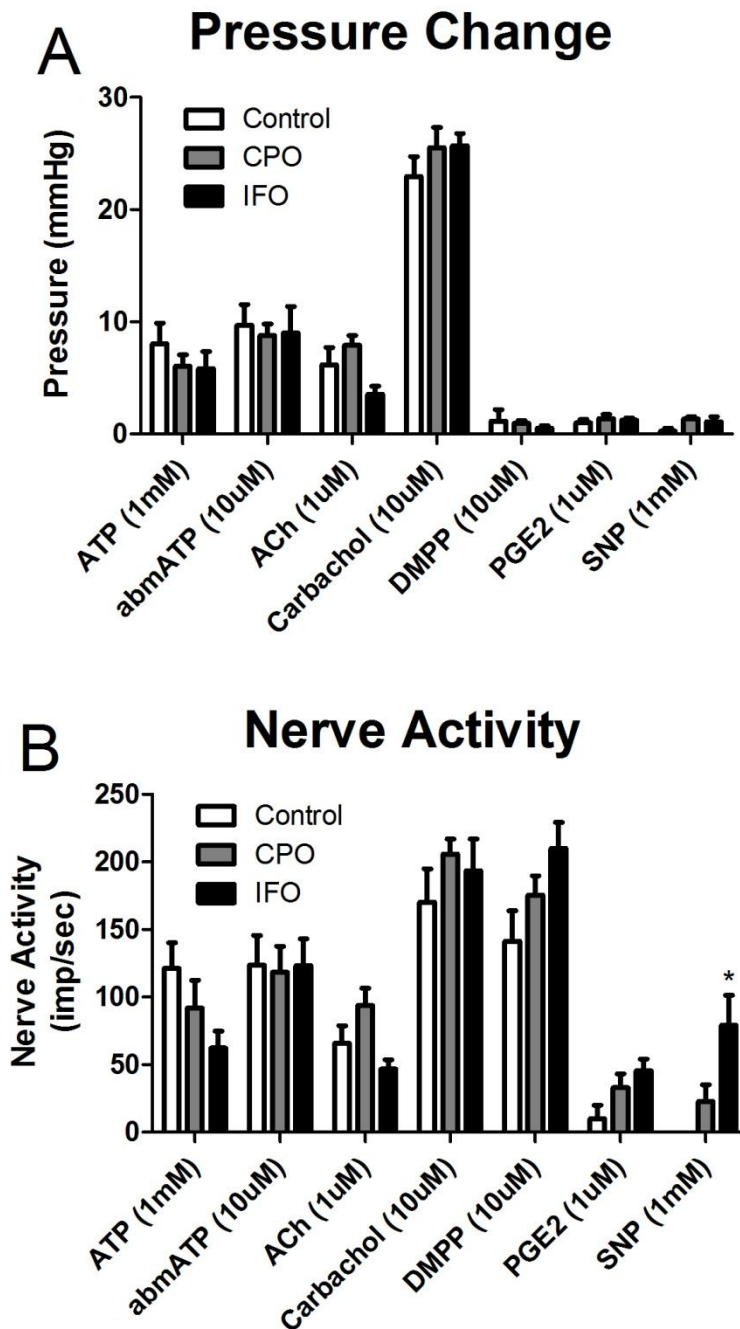


Figure 5.24: Effect of CPO or IFO treatment on intravesical pressure responses (A) and afferent nerve responses (B) to ATP (1mM), $\alpha\beta$ mATP (10 μ M), ACh (1 μ M), carbachol (10 μ M), DMPP (10 μ M) and PGE₂ (1 μ M). Data represented as mean \pm SEM (n \geq 5). Analysed by one way ANOVA with Dunnett post test (*p<0.05 vs control response for each drug).

In control bladders, nifedipine (1 μ M) significantly reduced the intravesical pressure responses to ATP (1mM), $\alpha\beta$ mATP (10 μ M) and carbachol (10 μ M) ($p < 0.05$) (**Figure 5.25-A**). The pressure responses to DMPP (10 μ M) were almost completely removed in the presence of nifedipine (1 μ M) but this was not significant given the initial responses were small to begin with. Despite the significant reduction in muscle contraction to these pharmaceutical agents, nifedipine only reduced the nerve activity in response to carbachol (10nM) ($p < 0.05$) (**Figure 5.25-B**).

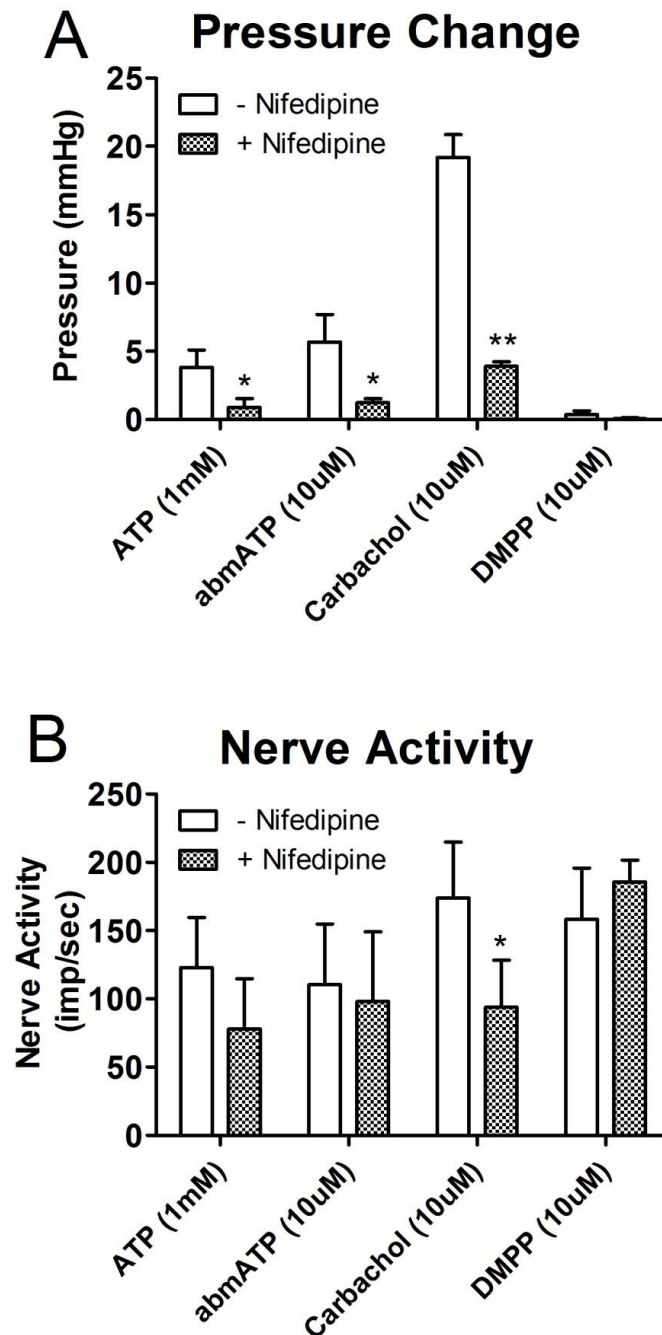


Figure 5.25: The effect of nifedipine (1 μ M) on control bladder pressure (A) and total nerve (B) responses to ATP (1mM), $\alpha\beta$ mATP (10 μ M), carbachol (10 μ M) and DMPP (10 μ M). Data represented as mean \pm SEM (n=4) and analysed by paired two-tailed t-test (* $p < 0.05$, ** $p < 0.01$ vs absence of nifedipine)

Table 5.1: Intravesical pressure and nerve activity responses to various agonists in the absence and presence of Nifedipine (1μM) in control and CPO treated bladders					
		Controls		CPO Treated	
		Pressure Response (mmHg)	Nerve Activity (imp/sec)	Pressure Response (mmHg)	Nerve Activity (imp/sec)
ATP (1mM)	-Nifedipine	3.8 \pm 1.3	123 \pm 37	5.7 \pm 1.3	114 \pm 46
	+Nifedipine	0.9 \pm 0.6	78 \pm 37	1.3 \pm 0.2	81 \pm 38
$\alpha\beta$ mATP (10 μ M)	-Nifedipine	5.7 \pm 2.0	111 \pm 44	6.7 \pm 1.4	126 \pm 48
	+Nifedipine	1.3 \pm 0.3	98 \pm 51	1.1 \pm 0.2	83 \pm 33
Carbachol (10 μ M)	-Nifedipine	19 \pm 2	174 \pm 41	23 \pm 2	224 \pm 25
	+Nifedipine	3.9 \pm 0.4	94 \pm 35	4.0 \pm 0.6	111 \pm 34
DMPP (10 μ M)	-Nifedipine	0.37 \pm 0.27	158 \pm 38	0.77 \pm 0.29	166 \pm 33
	+Nifedipine	0.07 \pm 0.06	188 \pm 16	0.19 \pm 0.12	235 \pm 22

The intravesical pressure responses to ATP (1mM), $\alpha\beta$ mATP (10 μ M), carbachol (10 μ M) and DMPP (10 μ M) were affected by nifedipine (1 μ M) to a similar extent in both control and CPO treated bladders (**Figure 5.26 & Table 5.1**). Specifically, contractions to ATP (1mM) were reduced in the presence of nifedipine (1 μ M) by 83 \pm 8% in controls and by 72 \pm 8% in CPO treated bladders. Responses to $\alpha\beta$ mATP (10 μ M) were similarly reduced by 75 \pm 5% and 78 \pm 8% in control and CPO treated bladders respectively. Carbachol responses in control and CPO treated bladders were reduced by 80 \pm 1% and 82 \pm 2%. The responses to DMPP were almost completely abolished, being reduced by 94 \pm 5% and 98 \pm 1% in control and CPO treated bladders respectively.

Nerve activity was less affected by nifedipine (1 μ M) than the pressure responses and again was not different between control and CPO treated tissues (**Figure 5.26 & Table 5.1**). Activity due to ATP (1mM) was reduced in the presence of nifedipine by 37 \pm 14% and 16 \pm 28% in control and CPO treated bladders. Similar results were seen in response to $\alpha\beta$ mATP (10 μ M) with nerve activity reduced by 18 \pm 13% and 38 \pm 5% in bladders from control and CPO treated mice respectively while carbachol responses were reduced by 49 \pm 10% and 54 \pm 10% respectively. The nerve activity responses to DMPP (10 μ M) were not reduced by nifedipine (1 μ M) but were actually increased by 34 \pm 26% and 50 \pm 15% in control and CPO treated tissues respectively.

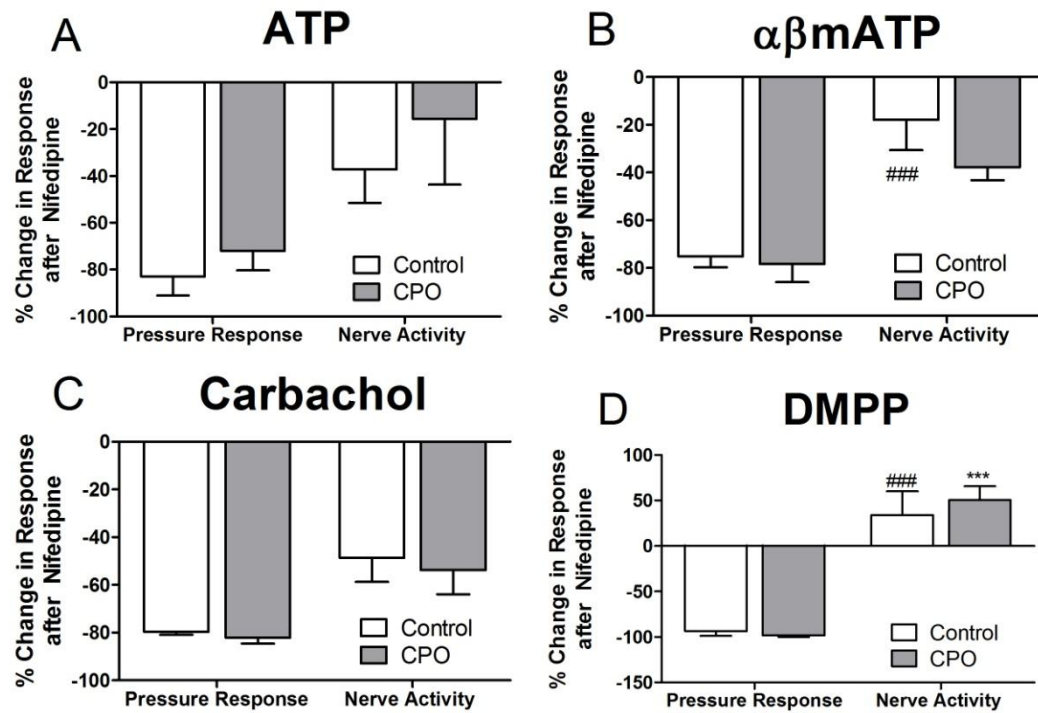


Figure 5.26: Percentage change of the intravesical pressure and nerve activity responses to ATP (1mM) (A), $\alpha\beta$ mATP (10 μ M) (B), carbachol (10 μ M) (C) and DMPP (10 μ M) (D) in the presence of nifedipine (1 μ M) in control and CPO (100mg/kg) treated bladders. Analysed by two-tailed, unpaired t-test (###p<0.001 vs control pressure change, ***p<0.001 vs CPO pressure change).

Mediator Release

Given the importance of urothelial mediators in bladder function and the results in Chapters 3 and 4, the release of ATP and ACh into the lumen of the bladders was examined. Luminal contents of the bladders during basal and stretch conditions in the absence and presence of the ATPase inhibitor ARL67156 (100 μ M) were analysed for total ATP and ACh content. The total amount of each mediator in each sample was calculated and the basal and stretch concentrations compared (**Figure 5.27**). Stretch induced ATP and ACh levels were not significantly different from basal levels. The presence of ARL67156 tended to increase ATP levels while decreasing ACh levels however these effects were not significantly different.

Treatment with CPO showed a trend towards decreased ATP release compared to controls (**Figure 5.28-A**). Basal and stretch ATP levels were reduced by 50% and 60% respectively, however this was not statistically significant. ACh levels were also slightly decreased after CPO treatment, but again not significantly (**Figure 5.28-B**).

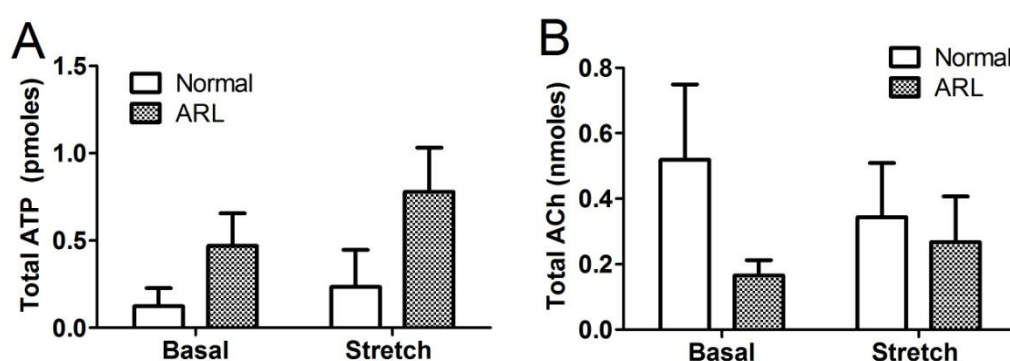


Figure 5.27: Total ATP (A) and ACh (B) in basal and stretch samples in the absence and presence of ARL67156 (100 μ M) from bladders of control mice. Data represented as mean \pm SEM (n=4). Analysed via paired two-tailed student's t-test.

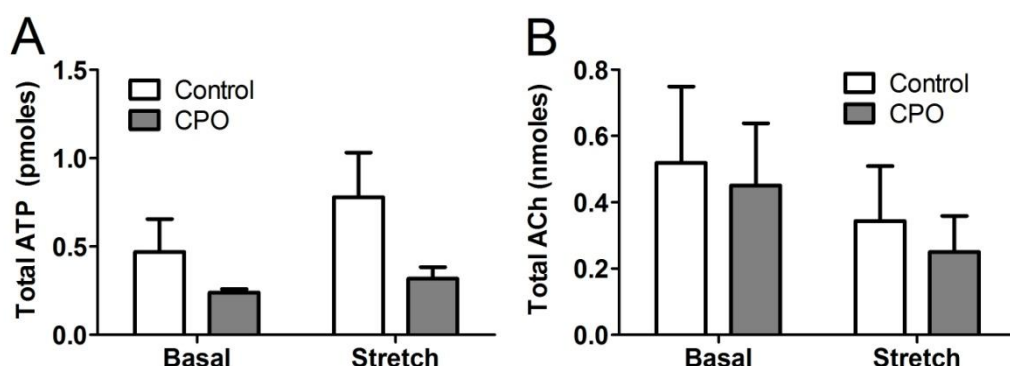


Figure 5.28: Total ATP (A) and ACh (B) in basal and stretch samples from bladders of mice treated with saline (control) or CPO (100mg/kg). Data represented as mean \pm SEM (n=4). Analysed via paired two-tailed student's t-test.

Response to Intravesical Instillation of Acrolein or CAA

Although the parent drugs appeared to affect nerve activity, it was not apparent what metabolite was causing the changes. Accordingly, intravesical instillation of acrolein or CAA was performed to determine if either metabolite had an effect on afferent nerve activity. The following experiments were performed opportunistically when untreated bladders were available from other researchers in our laboratory. Accordingly, the following results represent $n=1$ for each concentration tested. Untreated bladders were distended with 0.9% saline and then the distension repeated with saline containing acrolein ($1\mu\text{M}$ or $100\mu\text{M}$) or CAA ($1\mu\text{M}$). The muscle developed larger and more frequent phasic contractions during distension with $1\mu\text{M}$ acrolein compared to control distensions (**Figure 5.29-A**). Intravesical installation of acrolein ($1\mu\text{M}$) also caused an increase in total nerve activity at pressures above 20mmHg during distension (**Figure 5.29-B**). At higher concentrations, acrolein ($100\mu\text{M}$) also induced larger and more frequent spontaneous contractions (**Figure 5.30-A**), however total nerve activity was almost halved at pressures above 20mmHg during distension (**Figure 5.30-B**). Intravesical installation of CAA ($1\mu\text{M}$) caused an increase in total afferent activity during distension without affecting phasic activity in the muscle (**Figure 5.31**).

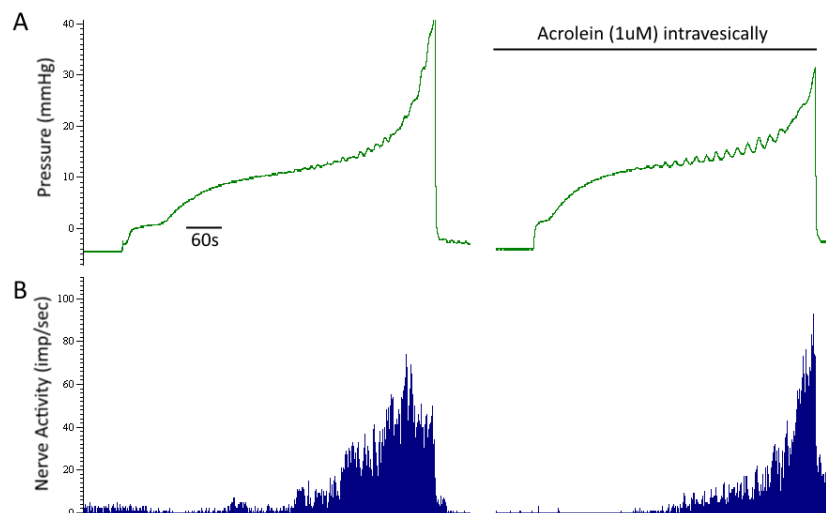


Figure 5.29: Representative trace of an untreated mouse bladder showing intravesical pressure (A) and nerve activity (B) during distension with 0.9% saline in the absence and presence of acrolein ($1\mu\text{M}$).

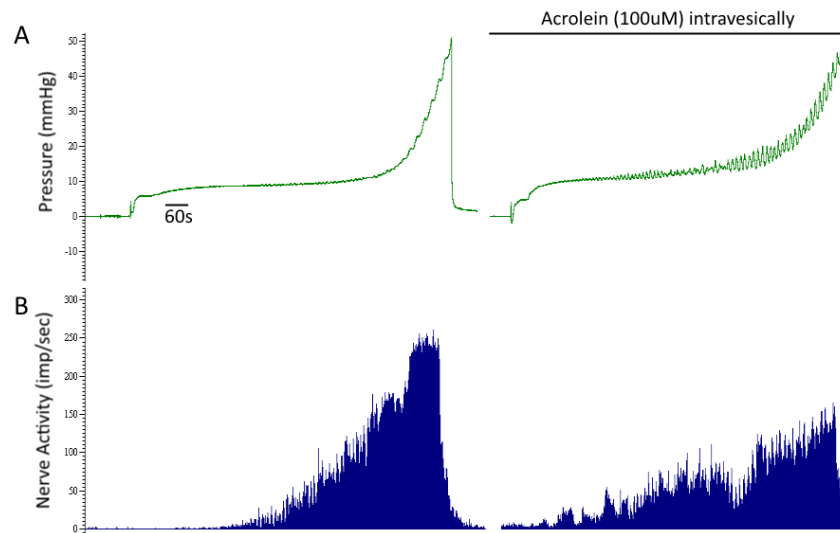


Figure 5.30: Representative trace of an untreated mouse bladder showing intravesical pressure (A) and nerve activity (B) during distension with 0.9% saline in the absence and presence of acrolein (100µM).

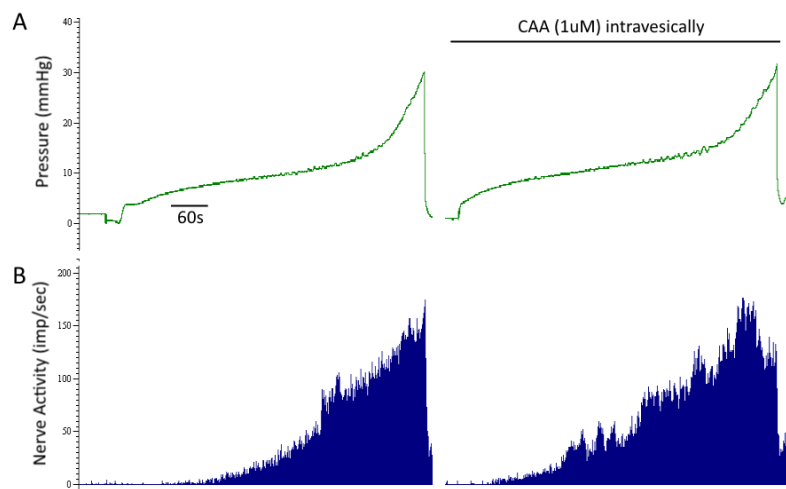


Figure 5.31: Representative trace of an untreated mouse bladder showing intravesical pressure (A) and nerve activity (B) during distension with 0.9% saline in the absence and presence of chloroacetaldehyde (CAA) (1µM).

5.4 DISCUSSION

The afferent nerves in the bladder are crucial for normal bladder sensations and function. They are responsible for sensing chemical and mechanical changes in the bladder and coordinating the control of storage and voiding functions (Kanai and Andersson, 2010). Changes to afferent nerve density, receptor expression, responsiveness or activation thresholds may be associated with bladder dysfunction such as neurogenic or idiopathic detrusor overactivity, painful bladder syndrome and the detrusor overactivity associated with bladder outlet obstruction (de Groat and Yoshimura, 2009). Cyclophosphamide and ifosfamide are known to cause lasting sensory changes in the bladder such as increased frequency and urgency of micturition, feelings of residual volume and pain.

The results described above show that as volume in the bladder increased, intravesical pressure and bladder sensory nerve activity increased. This is consistent with observations reported in other studies using the same technique (Daly et al., 2007, Rong et al., 2002) despite their use of faster filling rates of 100 μ l/min and 200 μ l/min respectively. The filling rate of 30 μ l/min used in this study was calculated to be a more physiological bladder filling rate given that cystometry studies in awake normal mice have shown an average voiding volume of 200 μ l every 380 seconds (equating to approximately 32 μ l/60sec) (Birder et al., 2002a). Despite the differences in filling rate used, the pressure and afferent firing responses were similar to these previous studies, with two filling phases and a non-linear relationship between pressure and afferent activity observed (Daly et al., 2007, Rong et al., 2002). The first phase of bladder filling (between 0-20mmHg) included a gradual increase in intravesical pressure and a marked increase in afferent firing while during the second phase (between 20-40mmHg) intravesical pressure increased quickly with a smaller increase in afferent activity. This non-linear relationship is best demonstrated in **Figure 5.14-B** and may be explained by the features and contribution of low and high threshold nerves which will be discussed in more detail below.

Does CPO or IFO Treatment Enhance the Activity of Afferent Nerves?

The results of the present study demonstrated that pre-treatment with CPO or IFO enhanced total afferent nerve firing during bladder distension in mice. This supports the results of previous studies discussed in the introduction of this Chapter and the theory that CPO and IFO induced bladder hyperactivity is due to increased afferent nerve activity. Although activity was enhanced at maximum distension, a greater enhancement was actually observed at lower intravesical pressures of around 15mmHg. An intravesical pressure of 15mmHg corresponds to the approximate micturition threshold in mice (Igawa et al., 2004). Therefore, it is possible that

the effects of CPO and IFO on afferent activity are most apparent during typical physiological filling.

A particularly relevant study performed in CPO treated rats also showed an increase in afferent nerve firing (Yu and de Groat, 2008). The study used an isolated rat bladder model and measured afferent activity in response to distension at 40 μ l/min and in response to electrical stimulation of the bladder. In rats pre-treated with CPO (100mg/kg) 17 hours earlier, afferent nerve activity was significantly increased in response to both distension and electrical stimulation. However marked variability was found between the afferent responses and this was thought to be at least partially due to variation in the number of fibres present in the afferent branch during recording. Accordingly, the present study goes further to investigate whether increased afferent activity after CPO or IFO treatment is due to increased firing of each fibre or rather an overall increase in fibre density.

Discrimination of the single units contributing to the total nerve response in each experiment revealed that both CPO and IFO enhanced the nerve activity per fibre. This result confirms that the increase in total activity seen was in fact due to CPO or IFO enhancing the firing rate of the afferent nerves. This also supports the previous findings of increased action potentials in isolated DRG neurones from CPO treated rats (Yoshimura and de Groat, 1999, Dang et al., 2008) and suggests that these findings are likely to be occurring in physiological conditions.

Given that both CPO and IFO metabolism produces the toxic metabolites acrolein and CAA, it is possible that the increased nerve activity is due to one or both metabolites present in the urine directly or indirectly affecting nerves. Intravesical instillation of acrolein (1 μ M) or CAA (1 μ M) into bladders during distension caused an increase in nerve activity. A higher concentration of acrolein (100 μ M) appeared to damage nerves causing reduced nerve activity. Acrolein also caused an increase in the frequency and amplitude of spontaneous contractions during distension while CAA did not. These observations came from a single experiment with each concentration so should be interpreted with caution. However, similar effects of intravesical acrolein have been reported previously where 0.003% acrolein increased A δ and C-fibre afferent activity and appeared to increase spontaneous contractions (Aizawa et al., 2011, Minagawa et al., 2012). No other studies have investigated the effect of intravesical instillation of CAA on bladder afferent nerves. In these preliminary investigations, the effects of acrolein on nerve activity were greater than CAA however they suggest that both metabolites are capable of increasing afferent firing during distension and further investigation would be warranted.

The metabolism of CPO and IFO was discussed previously. CPO metabolism occurs approximately 80% via an acrolein producing pathway and 20% via a CAA producing pathway whereas, IFO metabolism occurs by approximately 50% acrolein and CAA producing pathways. Initially, IFO was investigated at an equal dose to CPO (100mg/kg) to investigate the effect of less acrolein and higher CAA production on afferent nerve activity. No change in afferent nerve activity was seen after treatment with this lower dose of IFO (100mg/kg) despite a significant increase in activity after CPO treatment at the same dose (100mg/kg). Next, a higher and more clinically relevant dose of IFO was used (200mg/kg) and compared to changes induced by CPO (100mg/kg). At these doses it was assumed that there would be similar levels of acrolein present in the urine of both treatment groups while CAA levels would be higher in IFO treated animals. Despite the assumed differences in urinary metabolite concentration after CPO or IFO treatment, there was no difference between the nerve activity observed in either group. This supports the belief that acrolein is the major metabolite associated with CPO or IFO urotoxicity, however it does not rule out a role for CAA.

To investigate the potential role of CPO or IFO in bladder muscle function, the pressure–volume relationship during bladder filling and accommodation was assessed as an index of compliance. Treatment with CPO or IFO had no effect on bladder compliance during filling while causing an increase in total nerve activity. Bladder compliance during accommodation was also unchanged after CPO or IFO treatment. Despite total nerve activity being increased at 20mmHg in both CPO and IFO treated animals the nerves were able to adapt over time to a similar firing rate as in control animals. Previous studies in mice and rats have shown the same response pattern in pelvic afferents, demonstrating dynamic responses to graded bladder distensions and slow adaptation to a maintained stimulus or distension (Sengupta and Gebhart, 1994, Shea et al., 2000, Jung et al., 2012). What is interesting is that the nerves retained this ability to adapt after CPO or IFO treatment and they adapted to a similar level. However, physiological conditions would rarely require maintenance of a stable stimulus and rather the bladder volume and pressure would be continually changing. Accordingly, CPO and IFO appear to enhance the afferent response to the physiologically relevant graded stimuli.

Does CPO or IFO Treatment Enhance Activity of Low or High Threshold Afferent Nerves?

As discussed previously, two populations of afferent nerves exist in the bladder (low and high threshold) and they contribute differently to overall bladder sensations. The existence of both low and high threshold bladder afferents in the pelvic nerve has been well documented in

several species including mice (Habler et al., 1990, Sengupta and Gebhart, 1994, Daly et al., 2007, Rong et al., 2002). In mice, low threshold fibres have been reported to make up 70-80% of the total bladder afferent fibres (Daly et al., 2007, Rong et al., 2002). However, the present study found that 60% of all fibres were low threshold, a figure slightly lower than previous reports. It is possible that bladder compliance may have been affected by the faster filling rates used in the previous studies causing differences in afferent activation. This could explain the slight differences in the ratio of low and high threshold fibres seen in this study. Using a different technique in which the bladder was opened flat and stretched manually, 64% of fibres were found to be low threshold fibres (Jung et al., 2012) which is comparable to the results of the present study. A related study in the guinea pig bladder using a similar manual stretch technique showed that 75% of the stretch sensitive pelvic nerve afferents had low thresholds (Zagorodnyuk et al., 2006). This variation may reflect differences between species or differences between bladder filling and manual stretch methods. Nonetheless, all studies agree that low threshold fibres are more numerous than high threshold fibres.

Several studies have also reported that the low threshold nerves demonstrate a higher firing rate or greater magnitude of response than high threshold fibres (Jung et al., 2012, Daly et al., 2007, Shea et al., 2000). Specifically, Xu & Gebhart (2012) reported the average response of low threshold fibres to be approximately double the mean response of high threshold fibres at maximum stretch. Similarly, in this study, the activity of low threshold fibres was approximately 60% greater than that of the high threshold fibres at maximum distension.

As discussed earlier, two response patterns of low threshold stretch sensitive afferents have been reported in rats (Shea et al., 2000) and mice (Jung et al., 2012) and have been termed encoding and non-encoding fibres. In the present study, both filling patterns were observed in the low-threshold nerves and the non-encoding response was more common. All high threshold fibres however demonstrated an encoding pattern with activity increasing continuously up to maximum distension both in the present study and that by Xu & Gebhart (2012). The occurrence of these two different response patterns in low threshold fibres could potentially explain the non-linear relationship between pressure and afferent activity described above. All low threshold fibres increased in activity up to 10-15mmHg at which stage the more common non-encoding fibres started to plateau or even decrease in activity having the effect of flattening the pressure-response curve as can be seen in **Figure 5.19**. The firing rate of the less common encoding low and high threshold fibres then continued to increase at pressures above 15mmHg contributing to the more gradual increase in the pressure-response curve up to maximum distension.

Treatment with CPO or IFO enhanced the activity of the low threshold fibres but not the high threshold fibres. This is the first study to report the effect of CPO or IFO on activity in the distinct populations of low and high threshold bladder afferents. Rong et al., (2002) demonstrated a similar increase in stretch induced firing of low threshold fibres after exposure to $\alpha\beta$ mATP (1mM). Furthermore, in high threshold fibres $\alpha\beta$ mATP exposure led to a decrease in activation threshold and an increase in firing rate during distension. Xu & Gebhart (2012) also demonstrated sensitisation of low and high threshold fibres in response to a mixture of inflammatory mediators. In particular, the response threshold of the high threshold fibres was reduced after exposure to the mixture to a level comparable to that of the low threshold fibres. Interestingly, the response pattern of some non-encoding low threshold fibres changed after exposure to the inflammatory mediators exhibiting encoding responses up to maximum stretch.

In the present study separate mice were treated with saline, CPO or IFO and accordingly, it was not possible to determine whether the drugs lowered the activation threshold of high threshold fibres. However, the percentage of low threshold fibres did show an increased trend after CPO or IFO treatment suggesting that some of the high threshold fibres may have been sensitised becoming active at low threshold pressures. Similarly, it was not possible to determine if response patterns of specific low threshold fibres were altered by CPO or IFO treatment. Again the percentage of encoding fibres increased after treatment suggesting that some non-encoding fibres may have been altered by CPO or IFO. In addition, it was not possible to determine whether CPO or IFO treatment caused any silent afferents to become mechanosensitive. However, given that cystitis induced by intravesical instillation of acetic acid, turpentine oil or inflammatory mediators does appear to sensitise silent afferents, it is possible that the same would occur after CPO or IFO induced cystitis.

Typically, low threshold fibres are thought to contribute to normal micturition reflexes and high threshold fibres to painful sensations. However, given that low threshold fibres can encode stretch stimuli into the pain/noxious range (>15mmHg) and can sensitise, it appears possible they may also contribute to painful sensations. Accordingly, the increased activity of low threshold fibres after CPO or IFO treatment may explain the bladder hyperactivity (urgency, frequency), abnormal sensations (residual volume) as well as the painful sensations experienced by patients. Furthermore, high threshold fibres becoming active at lower threshold could also explain abnormal sensations of fullness and pain at lower bladder volumes.

The mechanism of how CPO or IFO causes sensitisation of the bladder afferent nerves is unknown. As discussed in Chapter 3, various mediators released by the urothelium such as

ATP and PGE₂ can activate and sensitise afferent nerves and levels of both these mediators was enhanced in the human urothelial cells in Chapter 3. Therefore, it is possible that increased ATP and/or PGE₂ from the urothelium may be causing afferent sensitisation and increased responses during bladder filling. Specifically, activation of the P2X3 receptors can sensitise bladder afferents (Rong et al., 2002) and excess urothelial ATP may be responsible for such an effect in CPO or IFO induced hyperactivity. However, the results in this Chapter did not demonstrate increased luminal ATP levels in the mouse. Accordingly, the increased afferent activity observed in this study does not appear to be associated with increased ATP release from the urothelium. This was unexpected as Smith et al., (2005) have previously reported increased ATP release from rat urothelium after CPO treatment. However, the model used a 50% higher dose of CPO administered chronically every three days for a total of three treatments. Therefore, the increased ATP release seen by Smith et al.,(2005) may be a long term effect of CPO or may be a result of greater urothelial changes due to a higher dose than used in the present study.

Interestingly in DRG from CPO pretreated rats, P2X3 receptors were up-regulated with 68% of pelvic neurones expressing these receptors (up from 40% in controls) suggesting *de novo* synthesis in neurones that do not normally express them (Dang et al., 2008). Furthermore, a greater number of neurones responded to purinergic agonists with more action potentials, greater current density and slower desensitisation kinetics. Accordingly, it appears that CPO not only increases P2X3 receptors in bladder afferents but also sensitises and enhances the activity of these receptors. Therefore, even normal levels of stretch induced urothelial ATP release could cause more afferents to be activated and their responses to be enhanced. ATP appeared to play a role in the increased afferent firing to rat bladder distensions after CPO treatment as purinergic antagonists significantly reduced the afferent firing at low intravesical pressure (10cm H₂O) (Yu and de Groat, 2008). The increased activity at pressures of 20cm H₂O and higher were not significantly affected by blocking the purinoceptors. Therefore, it is possible that increased urothelial ATP or enhanced responses to ATP may partly contribute to the increased nerve activity after CPO or IFO treatment. However, the results of the present study and those of (Yu and de Groat, 2008) suggest that there is another mechanism involved in the afferent sensitisation seen after treatment with these chemotherapeutics.

Intravesical administration of PGE₂ enhances the micturition reflex in mice increasing frequency of micturition and micturition pressure (Ishizuka et al., 1995b). Furthermore, an EP3 receptor antagonist reduced afferent firing in rats in response to bladder distension directly implicating PGE₂ in afferent responses to bladder filling (Su et al., 2008). Various EP receptor antagonists

have also been shown to attenuate CPO induced hyperactivity and pain in rodents (Chuang et al., 2012, Miki et al., 2011). These studies used a single high dose of CPO (200mg/kg and 300mg/kg respectively) to study the effect of PGE₂ on bladder function. The dose used in the present study (100mg/kg) was lower than in these previous studies and luminal samples were too small to allow measurement of PGE₂ content. However, initial results in Chapter 3 in human urothelial cells indicated that acrolein and CAA can both enhance PGE₂ release. Accordingly, it is possible that PGE₂ release from the urothelium may be contributing to the increased afferent response observed by activating or sensitising afferent nerves to mechanical stimulation.

Muscle and Nerve Response to Pharmacological Agents

Although nerve activity was increased at 20mmHg after CPO or IFO treatment, the firing rate in both treatment groups was able to accommodate over time to a level comparable to controls. This gave a fairly consistent baseline firing rate from which responses to various agonists could be assessed. It is acknowledged that the addition of various mediators in succession may lead to possible interactions in the tissue. When planning these experiments, care was taken to choose an order of addition of these agents that minimised possible interactions. Given the difficulty in setting up these experiments it was important to investigate as many agents as possible and determine potential avenues for further investigation. To overcome the potential limitation of drug interaction, the agents were always added in the same order in every animal and responses were allowed to come back to baseline before adding another agent. Some potential interactions have been discussed further below.

Afferent nerves responded to purinergic activation with fast increases in firing rate that peaked quickly and then recovered back to baseline within 10 minutes. The nerve responses to $\alpha\beta$ mATP (10 μ M) were greater than the responses to ATP (1mM). Both purinergic responses were associated with a similar increase in intravesical pressure, generally increasing at the same time and taking the same time to recover to baseline. This was expected as previous studies have reported similar responses to purinergic activation. Specifically, Yu & de Groat (2008) demonstrated in rats that ATP (2mM) elicited a bladder contraction and an associated burst of afferent firing. In guinea pig bladder, $\alpha\beta$ mATP (30 μ M) also evoked contractions and associated firing in stretch sensitive afferents (Zagorodnyuk et al., 2006).

Both muscle and nerve responses to $\alpha\beta$ mATP (10 μ M) were unchanged after treatment with CPO or IFO. Responses to ATP (1mM) were also unaffected by treatment and if anything showed slight decreases. The nerve results are unexpected as CPO treatment has been shown to increase the P2X3 density in afferent neurones and enhance the activity of these

receptors (Dang et al., 2008). However, these effects were seen after chronic CPO treatment with 3 doses of 100mg/kg each two days apart. Therefore, it is possible that purinergic enhancement does not contribute to the acute afferent sensitisation observed in the present study but rather comes into play during chronic irritation.

In addition, the muscle responses to purinergic agonists in the present study are also unexpected based on previous publications. Specifically, Mok et al., (2000) demonstrated in the house musk shrew that contractile responses of isolated bladder strips to β,γ -mATP were reduced by almost 50% after a single high dose of CPO (200mg/kg), however only at a concentration of 300 μ M. It is possible that the lower dose of CPO (100mg/kg) used in the present study did not cause sufficient bladder damage to reduce the purinergic response to the same extent. However, given that a slight reduction was observed, it is also possible that the responses to higher doses of ATP could have revealed a significant decrease in response.

In any event, the increased afferent activity observed in the present study does not appear to be due to enhanced responsiveness to purinergic activation and although this mechanism may contribute to changes after chronic CPO or IFO treatment, there appears to be another mechanism contributing to enhanced afferent responses at least during the acute stage of urotoxicity. However, the results of the present study do not rule out the possibility that increased local urothelial ATP release in association with afferent nerve endings could contribute to the enhanced activity. Accordingly, performing distensions in the presence of a purinoceptor antagonist could shed light on whether the enhanced activity in CPO or IFO treated bladders is due to excess ATP.

Muscarinic receptor stimulation with ACh (1 μ M) or carbachol (10 μ M) also caused an increase in nerve firing but the responses were slower to peak and took much longer to recover back to baseline (approximately 45 minutes) than purinergic responses. The carbachol responses were also greater than the responses to ACh. Muscarinic nerve responses were also associated with a similar increase in intravesical pressure, generally increasing at the same time and taking the same time to recover to baseline.

Bladder muscle contraction in response to muscarinic receptor stimulation is well known whereas the effect on afferent nerve activity is still unclear. Intravesical administration of a muscarinic receptor agonist oxotremorine-M did not stimulate afferent nerve activity in the absence of bladder distension but increased the firing induced by bladder distension (Yu and de Groat, 2010). However, Daly et al., (2010) found that muscarinic receptor stimulation during bladder distension significantly inhibited afferent firing and this was independent of muscle tone.

Accordingly, the effects of muscarinic receptor stimulation on afferent firing are still unclear. The results from the present study indicate the presence of afferent activity in response to muscarinic agonists, however whether this is solely due to muscle tone is uncertain. The effect of uncoupling muscle tone from afferent activity using nifedipine is discussed below.

In the present study, CPO or IFO pre-treatment did not significantly affect any of the responses to muscarinic receptor stimulation. Pressure and nerve responses to carbachol were marginally increased in CPO and IFO treated bladders whereas responses to ACh were slightly increased after CPO treatment and slightly decreased after IFO treatment. No studies have previously looked at the muscarinic response of nerves in CPO or IFO treated tissues. However for muscle, these results are opposite to what has been previously reported in isolated bladder strips from rats. Two studies have shown reduced contractions of isolated bladder strips from CPO or IFO treated animals to both carbachol ($>1\mu\text{M}$) and KCl (Macedo et al., 2011, Giglio et al., 2005). The reduced responsiveness to KCl observed previously also suggests that a general reduction in muscle contractility may be contributing to the reduced responses to carbachol rather than a specific muscarinic depression. In the present study, neither CPO nor IFO pre-treatment caused any significant change to muscarinic responses in the muscle or the nerve. It is possible that the differences between the previous studies in isolated bladder strips and the present study in whole bladder could be due to greater preservation of efferent and afferent innervation in whole bladder tissue, measuring intravesical pressure as opposed to direct contractile force of strips or even due to application of pharmacological agents on the serosal surface of the whole bladder as opposed to both urothelial and serosal sides of a tissue strip.

The nerves also demonstrated a large increase in firing rate in response to nicotinic receptor stimulation with DMPP ($10\mu\text{M}$) which peaked quickly and recovered within 10-15 minutes. However, this response was not associated with an increase in intravesical pressure. Therefore, the responses to DMPP are a good measure of nerve activity separate from muscle tension.

Nicotinic receptors are present in nociceptive, sensory neurones and nicotine can induce inwards currents in rat DRG neurones (Haberberger et al., 2004). Furthermore, mRNA expression of various nicotinic receptor subtypes has been recently shown in bladder sensory nerves in mice (Nandigama et al., 2013). In particular, the $\alpha 3$ -containing subtype was expressed in 69% of sensory nerves. Patch clamp recordings of rat bladder sensory DRG neurones demonstrated nicotine ($300\mu\text{M}$) induced inward currents (Masuda et al., 2006). Afferent nerve responses have also been reported previously in multi-unit nerve recordings in

rats where intra-arterial injection of nicotine caused bladder afferent nerve activity to increase without any increase in bladder pressure (Kontani et al., 2009).

Furthermore, nicotinic receptor mediated sensitisation mechanisms have been shown to be present in the bladder and can induce bladder hyperactivity. Specifically, a high dose of intravesically administered nicotine caused overactivity in anaesthetised rats decreasing the ICI and this could be prevented by pre-treatment with capsaicin or a nicotinic antagonist (Masuda et al., 2006). Beckel & Birder (2012) also demonstrated a functional role of nicotinic receptors on afferent nerve endings below the urothelium. Specifically, they used protamine sulphate to disturb the urothelial barrier and allow intravesically applied agents to pass into the underlying tissue. Before protamine sulphate, intravesical nicotine had an inhibitory effect on bladder reflexes decreasing urinary frequency. However, after protamine sulphate intravesical nicotine caused excitation of reflexes and subsequent overactivity suggesting that the urothelium releases an inhibitory mediator in response to nicotinic activation and that its removal then uncovers a direct stimulatory effect on afferent nerves.

After CPO and IFO treatment in the present study, muscle responses to nicotinic stimulation with DMPP did not change and while afferent activity showed an increased trend, the differences were not significant. This is the first study to investigate the nicotinic response of afferents after CPO or IFO treatment. Given that DMPP does not affect muscle tension, the nerve effects appear to be directly due to changes in nerve function. Therefore, the increased response to nicotinic receptor stimulation may be a result of increased excitability of the afferent nerves. This result supports the hypothesis that the increase in total nerve activity after CPO or IFO treatment is also due to sensitisation of the afferents lowering their activation threshold and increasing their firing rate to stimuli.

In the present study, PGE₂ application induced a slight increase in muscle tension accompanied with bursts of afferent activity. These results support previous studies such as those reviewed by Maggi, (1992) including the finding that topical application of prostanoids to a partially filled rat bladder induced a small tonic-type increase in bladder tone and a series of reflex bladder contractions mimicking the micturition reflex. Although direct nerve activity was not measured, the appearance of a series of micturition type contractions is suggestive of bursts of afferent activity stimulating the effects.

As outlined above, CPO or IFO pre-treatment had no effect on the muscle response to PGE₂ administration. However, both CPO and IFO treated bladders demonstrated a trend towards enhanced nerve activity in response to PGE₂. This is the first study to look at the effect of CPO

or IFO on afferent nerve responses to PGE₂ whereas most previous studies have only looked at a role for PGE₂ in the development of bladder hyperactivity to these chemotherapeutics. There appears to be a role for PGE₂ in sensitisation of afferent nerves to CPO as various EP receptor antagonists have also been shown to prevent the hyperactivity and pain (Chuang et al., 2012, Miki et al., 2011). The expression of the EP receptors is changed in bladders from rats treated with CPO showing increased EP4 expression, decreased EP1 expression and no change in EP2 or EP3 (Chuang et al., 2012, Chuang et al., 2010). The whole bladders were used in these studies so it is difficult to say what affect expression changes would have on function, however it is possible that altered receptor density could lead to altered responses to PGE₂. The present study indicates that the afferent nerves may have an altered response to PGE₂ after CPO and IFO treatment and this may be due to receptor expression changes or a change in excitability to receptor activation.

In untreated bladders the NO donor SNP had no effect on muscle or nerve responses which is similar to previous findings (Yu and de Groat, 2013). However, the present study demonstrated that administration of SNP in CPO or IFO treated bladders increased the frequency and firing rate of the bursts of afferent nerve activity induced by PGE₂. Furthermore, SNP caused a very slight increase in intravesical pressure in the treated bladders. This is unexpected given that several studies have reported NO as having an inhibitory effect on bladder hyperactivity induced by CPO (Yu and de Groat, 2013, Andersson et al., 2011). Specifically, blocking NO production in CPO treated rats caused an increase in the micturition frequency, but not in untreated animals (Andersson et al., 2011). In addition, application of a NO donor also caused inhibition of the afferent nerve activity during bladder distension after CPO treatment without significantly reducing maximum intravesical pressure (Yu and de Groat, 2013).

However, the present study used SNP after PGE₂ application which meant these two mediators may have been interacting. In rabbit bladder damaged by ischemia, the nitric oxide donor L-arginine caused a contraction that could be blocked by a COX-2 inhibitor, a response not seen in control bladders (Masuda et al., 2010). In addition, levels of PGE₂ present in tissues were higher after L-arginine in ischemia damaged bladder but not controls. This suggests that COX-2 expression is increased after ischemia and NO activates the enzyme to produce PGE₂. This mechanism may explain the results seen in the present study wherein an NO donor caused a slight increase in bladder pressure and an increase in nerve activity induced by PGE₂. These effects were only seen in CPO and to a greater extent IFO treated tissues and may be due to NO increasing PGE₂ production or enhancing the effects of previously administered PGE₂.

The Effect of Muscle Tone on Afferent Firing

As discussed above, the afferent nerve responses to ATP (1mM), $\alpha\beta$ mATP (10 μ M) and carbachol (10 μ M) were accompanied by similar increases in intravesical pressure suggesting that the nerve and muscle responses to purinergic and muscarinic activation are linked. The coupling of muscle tone to afferent firing is also supported by the phasic increases in intravesical pressure and corresponding afferent firing rate observed in the present study. Accordingly, the effect of muscle tone on the afferent responses was investigated by blocking muscle contraction with nifedipine (1 μ M). The effect of CPO or IFO on the contribution of muscle tone to afferent responses was also of interest.

In control bladders of the present study the phasic nerve activity mimicked the phasic muscle activity. In the presence of TTx (1 μ M) the phasic muscle contractions persisted but the nerve responses were abolished. This clearly demonstrates that afferent nerve activity can be influenced by muscle tension and supports previous findings (McCarthy et al., 2009, Zagorodnyuk et al., 2007). Specifically, Zagorodnyuk et al., (2007) demonstrated that some stretch-sensitive bladder afferents in the guinea pig show occasional bursting activity associated with spontaneous muscle contractions. In addition, McCarthy et al., (2009) observed spontaneous bladder contractions in both normal and spinal cord transected mice that were TTx insensitive, but could be blocked with nifedipine. The contractions were larger and less frequent in spinal cord transected mice and were associated with corresponding increases in afferent nerve activity. The results of the present study demonstrated that treatment with CPO or IFO did not significantly affect the frequency or amplitude of spontaneous contractions during filling or accommodation of the bladder. Furthermore, the nerve activity associated with the spontaneous contractions was not different after CPO or IFO treatment. The spontaneous contractions seen after CPO or IFO treatment will be discussed further in the next Chapter.

Nifedipine reduced the intravesical pressure responses to ATP and $\alpha\beta$ mATP while nerve responses were not reduced significantly. This suggests that the nerve responses to purinergic activation are almost completely due to direct activation of afferents rather than indirect mechanical activation due to muscle contraction. This is expected given that afferent nerves express purinergic receptors and activation causes neurone depolarisation triggering action potentials (Dang et al., 2008). The effect of nifedipine on purinergic responses was the same in controls and CPO treated bladders, reducing both the pressure and nerve responses to a similar extent. Accordingly, the contribution of muscle tone to the afferent nerve response is the same after CPO treatment.

Both the pressure and nerve responses to carbachol (10 μ M) were significantly decreased after the addition of nifedipine (1 μ M). However, nifedipine reduced pressure responses by approximately 80% while afferent responses were only reduced by 50%. This suggests that the afferent response to muscarinic receptor stimulation is largely due to muscle tone during the associated contraction. However, the muscle and nerve responses were not reduced to the same extent suggesting there are other muscarinic effects influencing nerve activity. It is unlikely that carbachol had any direct effect on afferent nerves as previous studies have demonstrated a lack of afferent firing in response to muscarinic agonists (Daly et al., 2010, Yu and de Groat, 2010). A possible explanation is that carbachol activated muscarinic receptors on the urothelial cells causing the release of excitatory mediators such as ATP and PGE₂ (Nile and Gillespie, 2012, Yokoyama et al., 2011) causing indirect activation of the afferent nerves. In terms of muscle tone contributing to afferent nerve response, both control and CPO groups demonstrated similar reductions in pressure and nerve responses after nifedipine. Again, supporting that in CPO treated bladders the relationship between muscle tone and nerve responses is unchanged.

The small pressure increases in response to DMPP were abolished in the presence of nifedipine, whereas the nerve responses tended to increase. This is an unexpected result and the mechanism behind this observation is not apparent. The same reduction in muscle responses and increase in nerve responses were seen in the control and CPO treated bladders. Therefore, the effect of muscle contraction on nicotinic induced nerve responses was unchanged by CPO treatment.

Conclusions

CPO and IFO treatment enhanced total nerve activity in the mouse bladder during graded distensions and this was due to enhanced activity of the low threshold fibres. Lowering of the activation threshold of some high threshold fibres may contribute to the increased nerve activity. In addition, an increased ability of low threshold fibres to encode into noxious ranges of bladder pressure may also be involved in the enhanced activity. This is the first study to report the effects of CPO and IFO on low and high threshold pelvic sensory nerves in the mouse bladder. The increased afferent activity does not appear to be due to enhanced responses to ATP, ACh or PGE₂ but rather appears to be due to sensitisation of the nerves making them more responsive to distension.

CHAPTER 6: Effects of Systemic Cyclophosphamide or Ifosfamide Treatment on Isolated Whole Bladder Function in Mice

6.1 INTRODUCTION

Afferent activity in the bladder is important for normal function. As discussed in the previous Chapter, alterations in afferent activity have been linked to various overactive and painful bladder disorders. Various factors can affect the activity of the afferent nerves including mediators released from the urothelium as well as detrusor muscle tone and spontaneous contractions. This study aimed to further investigate potential sources of afferent overactivity after CPO or IFO treatment by examining urothelial mediator release, detrusor contractility and efferent nerve function.

Comparison of Human and Mouse Bladder Function

Contraction of human and pig bladder is attributed almost completely to the release of ACh from parasympathetic nerves which act via the M3 receptor (Chess-Williams, 2002, Fetscher et al., 2002, Yamanishi et al., 2000, Sellers et al., 2000). Mouse urinary bladder responses differ from those of human or pig in that contractions are mediated by two transmitters acting on different receptors. In mice, the parasympathetic nervous system co-releases ACh and ATP to cause contraction (for review see Burnstock (2007)). Detrusor contraction to ACh has been shown to be mediated primarily by the M3 receptor in every species investigated (Uchiyama and Chess-Williams, 2004). This has also been confirmed in mice by Matsui et al., (2000) who found that M3 receptor knockout mice develop urinary retention. In addition, carbachol induced contractions of the bladder were reduced in M3 receptor knockout mice to only 5% of those seen wild type mice despite having comparable responses to KCl. Furthermore, in isolated mouse bladder strips various muscarinic receptor antagonists demonstrated binding affinities consistent with the M3 receptor being the primary mediator of direct muscarinic bladder contraction (Choppin and Eglen, 2001).

Although ATP is co-released with ACh from parasympathetic nerves in humans, it appears to have very little contribution to nerve mediated contraction however, a purinergic component emerges in various lower urinary tract disorders (Ford et al., 2006). The P2X1 is the most abundant P2X receptor subtype in the adult human bladder (O'Reilly et al., 2001) and may be up regulated in disease contributing to a purinergic component of nerve mediated contraction. In mouse, the ATP contraction is also thought to be mediated by the P2X1 receptor (Vial and Evans, 2000, Young et al., 2008). Immunoreactivity for the P2X1 receptor was found in smooth muscle of the mouse bladder while contractions to P2X agonists were abolished in P2X1 receptor deficient mice. Similarly, nerve induced responses in P2X1 knockout mice were

mediated solely by muscarinic receptors with no purinergic component remaining (Vial and Evans, 2000).

Control of human and pig bladder relaxation during filling is primarily due to noradrenaline release from sympathetic nerves acting on β 3-ARs in the detrusor (Badawi et al., 2007, Yamanishi et al., 2002d). The nonselective β -AR agonist isoprenaline has been shown to decrease micturition pressure and increase the time taken to empty the bladder in anaesthetised mice (Zhou et al., 2010). The isoprenaline induced relaxation of isolated mouse bladder strips was antagonised by a β 2-AR antagonist (Wuest et al., 2009). However, functional β 3-ARs have also been reported with selective β 3-AR agonist causing relaxation of mouse bladder strips (Deba et al., 2009).

The Effect of CPO or IFO on Bladder Function in the Rodent

Humans experience bladder pain and dysfunction after treatment with CPO or IFO. Rodents experience similar symptoms making them a useful animal model for studying the effects of these drugs. A single intraperitoneal injection of cyclophosphamide is sufficient to induce urotoxicity in mice and many of the typical features of urotoxicity appear within 24 hours after injection including pain, urinary frequency and activation of inflammation (Auge et al., 2013, Leventhal and Strassle, 2008). Rodents treated with CPO or IFO demonstrate typical urotoxic storage symptoms including increased urinary frequency and decreased contractile force leading to decreased voiding pressure (Okinami et al., 2014, Macedo et al., 2011, Boudes et al., 2011, Everaerts et al., 2010b, Wang et al., 2008b, Kageyama et al., 2008, Juszczak et al., 2007, Hu et al., 2003). In addition, animals also demonstrated enhanced intravesical pressure during filling and reduced bladder capacity. However, very little is known about what contributes to these functional changes in the bladder.

Maximum contraction to carbachol was reduced by approximately 45% in isolated bladder strips from CPO treated house musk shrew without any effect on muscle contractility to KCl (Mok et al., 2000). However, isolated bladder strips from rats with IFO-induced cystitis showed decreased contractile force development to both KCl and carbachol (Macedo et al., 2011). Similarly, in isolated bladder strips from rats with CPO induced cystitis, the maximum contractile effects to both carbachol and KCl were approximately half those of the control animals (Giglio et al., 2005). Interestingly, in the presence of L-NNA to block the production of NO, the responses to carbachol were increased to be similar to control responses. Levels of eNOS were significantly increased in the urothelium of CPO treated animals suggesting that the reduced contractions to carbachol were due to a cholinergic interaction causing NO release from

urothelial cells (Giglio et al., 2005). This was supported by Andersson et al., (2008) who showed that reduced cholinergic contractions in CPO inflamed bladders were partially restored by removal of the mucosa. In another study, the urodynamics of anaesthetised rats found that CPO treatment caused decreased voiding volume which could be normalised by the M3 antagonist 4-DAMP (Andersson et al., 2011). Furthermore, blocking NO production with L-NAME significantly enhanced the frequency of micturition in CPO treated rats with no effect in controls. Therefore, the CPO induced changes to bladder function involve enhanced NO production and alterations in muscarinic effects and interaction between the two systems seem likely.

Muscarinic receptor expression also appears to be affected by CPO treatment (Giglio et al., 2005). Using Western blotting and immunohistochemistry, all five (M1-5) receptor subtypes were found in both normal and CPO treated rats. However, the expression of M5 receptors was significantly increased in the smooth muscle and particularly the urothelium after CPO treatment. This suggests the changes in muscarinic responses in CPO induced cystitis are most likely due to enhanced M5 receptors on the urothelium leading to increased NO release.

Even less is known about the changes in response to purinergic contractions of the bladder after CPO or IFO treatment, with most of the effects attributed to sensory changes. However, in isolated bladder strips from CPO treated animals contractile responses to the P2X receptor agonist β,γ -methylene ATP (β,γ -mATP) were reduced by almost 50% without any effect on KCl induced contractions (Mok et al., 2000). Kageyama et al., (2008) found a loss of binding sites to [3H]- $\alpha\beta$ -mATP without a change in K_d suggesting that a down-regulation in P2X receptors may be responsible for the reduced P2X induced contractions.

Although these studies provide evidence of post-junctional alterations of the P2X and M3 receptors causing decreased contractile responses to exogenous agonists, little is known about the effect of CPO or IFO on efferent nerve induced responses.

Evaluation of bladder function

Clinically, the coordination of the bladder is evaluated by assessing the pressure to volume relationship during filling and pressure and urine flow rate during emptying. Many of the studies into bladder function use bladder muscle strips which are functionally limited to measuring contraction or relaxation in one plane. Recently, an isolated whole bladder model in mice was developed to offer a more physiological option to study bladder function (Fabiya and Brading, 2006). This model involves using a whole, isolated bladder from the mouse which maintains

intact urothelium, interstitial cells, detrusor and intramural ganglia and allows measurement of volume-pressure relationships. The whole bladders were distended submaximally and allowed to equilibrate for one hour to provide a stable intravesical tension from which to investigate the effects of various exogenous agonists as well as electrical stimulation of the intramural nerves releasing endogenous transmitters. The model was found to be viable and robust and has been used in the work forming this Chapter in order to study the effects of CPO or IFO on the function of the whole mouse bladder. The parameters tested were altered to suit the aims of the present investigation.

Aims

The aim of the present study was to investigate the function of the whole mouse bladder after treatment with CPO or IFO. Specific aims were:

- To investigate the effect of CPO or IFO treatment on spontaneous bladder contractions;
- To determine the release of ATP, ACh and PGE₂ from the urothelium and serosa of bladders treated with CPO or IFO;
- To determine the whole bladder response to electrical field stimulation of intramural nerves after CPO or IFO treatment;
- To assess the relaxatory adrenergic response of the whole bladder after treatment with CPO or IFO.

6.2 METHODS

Animals

Young adult male C57/6B mice were treated and sacrificed as outlined in the previous Chapter.

Isolated Bladder Experiments

The pelvis was placed into a bath of Krebs solution for setup as described in the previous Chapter. However, in this setup, a three way catheter was inserted through the urethra and secured in the bladder using silk suture. One port of the catheter was attached to an infusion pump to fill the bladder, another to a two-way outflow tap to allow emptying of the bladder and the third to a pressure transducer to measure intravesical pressure. The pressure transducer was attached to a Neurolog headstage (NL100, Digitimer Ltd, UK) connected to a pressure amplifier (NL108, Digitimer Ltd, UK) which amplified the pressure signals before they were passed to a Micro1401 analogue to digital interface (Cambridge Electronic Design, UK) and then visualised on a computer using Spike2 software (version 7.1, Cambridge Electronic Design, UK).

The ureters were ligated to prevent leakage of intravesical fluid. The bladder and urethra were dissected from the surrounding tissue and placed in a 10mL organ bath containing Krebs solution at 37°C, gassed with 95% O₂ - 5% CO₂. The bladder was distended with the infusion pump containing isotonic saline (0.9%) solution at 30µl/min to a maximum pressure of 40mmHg to check the viability of the catheterisation. The infusion pump was stopped and the outflow tap was opened to allow complete evacuation of the intravesical contents of the bladder. **Figure 6.1** shows the final setup of the isolated bladder. Baseline pressure when the bladder was empty was subtracted from the pressures during distension to give the change in pressure.

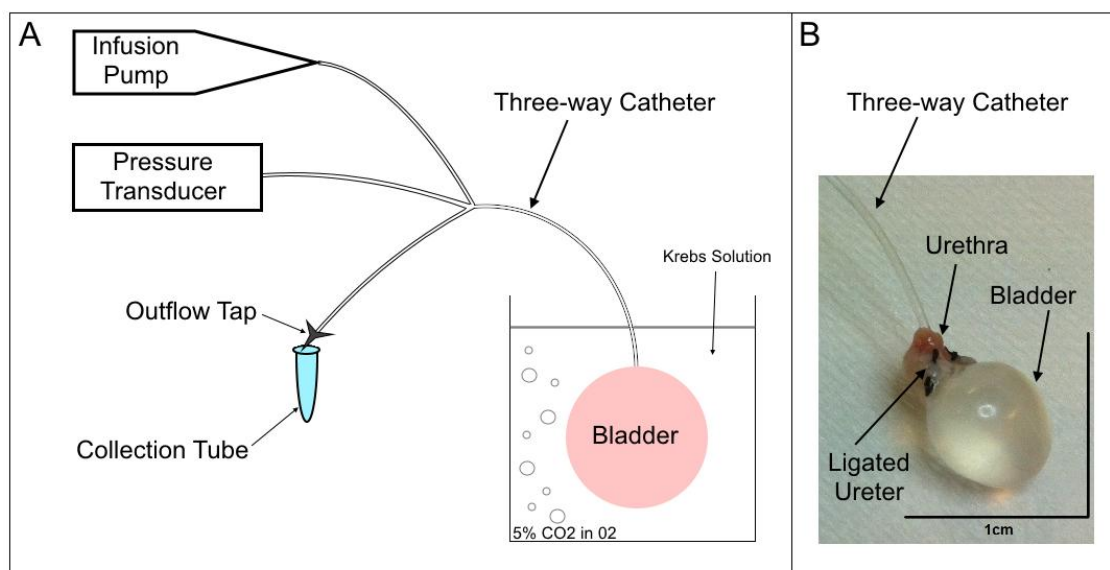


Figure 6.1: Schematic (A) of the isolated mouse bladder set up showing the bladder connected to the three-way catheter and a calibrated photograph (B) of a mouse bladder connected to a three-way catheter.

Observation of Inflammation

During dissection and setup, visual and microscopic observations of bladder inflammation were recorded. Specifically, the area and extent of haemorrhage was noted as well as the presence of visible vasculature in the bladder wall. Control bladders tended to be a consistent milky white colour with no visible vasculature, while the bladders treated with CPO or IFO tended to have patches of redness with visible vasculature as per the typical images shown in **Figure 6.2**.

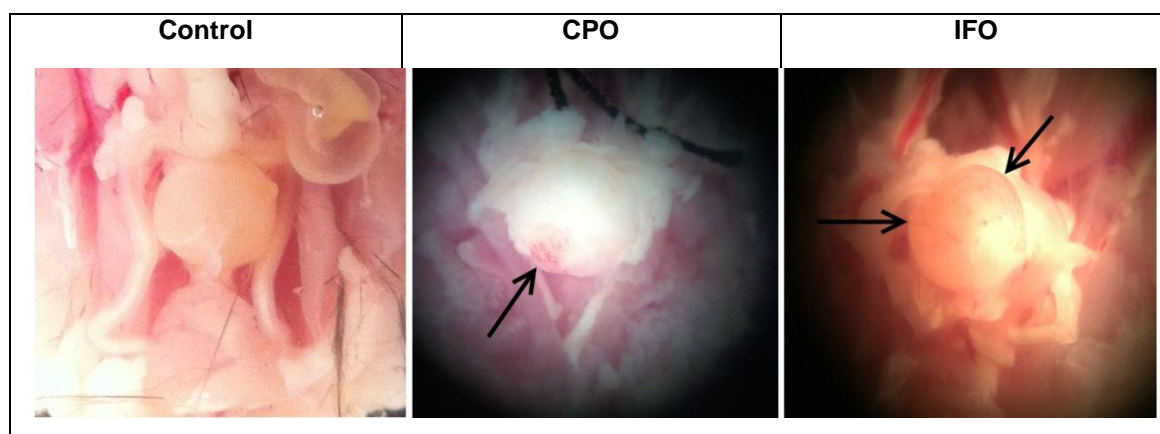


Figure 6.2: Typical images of control, CPO and IFO treated bladders with arrows indicating areas of inflammation and increased visible vasculature in treated bladders.

Measurement of Urothelial Mediators

Once the preparation had been successfully set up, samples were collected for measurement of ATP, ACh and PGE₂. The bath was washed and 8mL of fresh Krebs added, the outflow tap closed and the infusion pump turned on to start filling the bladder. The bladders were filled to a set pressure of 20mmHg. This was to ensure that each bladder received the same mechanical stimulation and reduced variability due to bladder size. Once the intravesical pressure reached 20mmHg, the infusion pump was turned off and the outflow tap opened. The luminal contents of the bladder were collected in a tube on ice and were stored as soon as possible after collection at -20°C. A sample of the Krebs in the bath in contact with the bladder serosa was also collected and stored at -20°C for later analysis. This process was repeated twice more to obtain luminal and serosal samples in triplicate with 10 minutes recovery between each distension. The luminal and serosal samples were then used in the various assays described in Chapter 2 for quantification of ATP, ACh and PGE₂ levels.

Electrical Field Stimulation

To study the efferent nerve response, the bladder was filled to a set pressure of 20mmHg and allowed to stabilise for an hour. The muscle then had a stable baseline tension from which to determine the effect of electrical field stimulation (EFS) by placing platinum electrodes either side of the bladder (**Figure 6.3**). The bladder was electrically stimulated (40 volts and 0.2ms pulse-width) delivered as a 5 second train every 100s, at frequencies of 5Hz, 10Hz and 20Hz. The tissues were stimulated at each frequency until stable responses were obtained. Initial responses for each frequency were obtained followed by responses at 20Hz only in the presence of 100μM L-NNA (NOS inhibitor), 1μM atropine (muscarinic antagonist) and 10μM alpha,beta-methylene ATP (αβmATP) (desensitises P2X purinoceptors) to investigate the contribution of NO, ACh and ATP to the contraction. In the previous Chapter, αβmATP (10μM) was used as an agonist, however after the initial response the P2X receptors are quickly desensitised and do not respond again for up to 30 minutes enabling it to block a further P2X mediated response (Fabiya and Brading, 2006). In this experiment, αβmATP (10μM) was present in the bath for at least 15 minutes before EFS was performed to ensure desensitisation of the P2X receptors. Responses to EFS in CPO or IFO treated tissues were compared to controls.

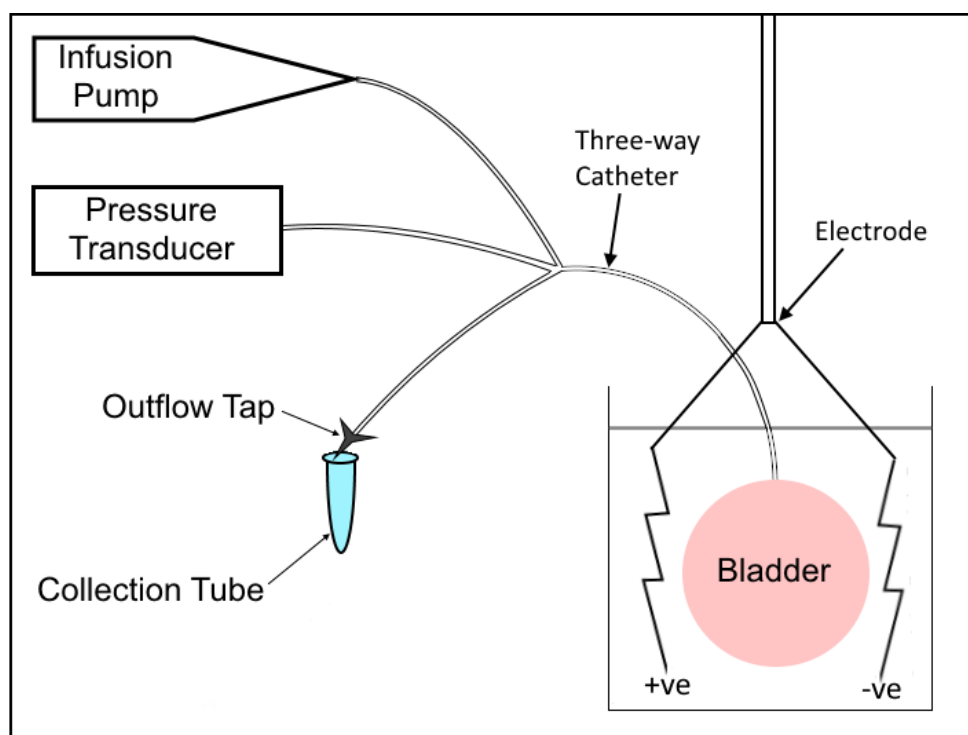


Figure 6.3: Schematic of the isolated mouse bladder electrical field stimulation set up showing the bladder connected to the three-way catheter between a positive and negative electrode for stimulation of the whole bladder.

Response to Pharmacological Agents

To study the effects of drugs on muscle tension, the bladder was filled to a set pressure of 20mmHg and allowed to stabilise for an hour. The muscle then had a stable baseline tension from which to determine the effect of drugs. Isoprenaline (non-selective β -adrenoceptor agonist) was then added to the bath cumulatively (1ng - 100 μ g) in half log intervals and the effect on muscle tension was calculated as the change in intravesical pressure from baseline.

Spontaneous Contractions

During bladder distension, phasic intravesical pressure increases were observed as the overall pressure increased (**Figure 6.4**). These spontaneous contractions demonstrated consistent frequency and amplitude within a bladder preparation (**Figure 6.4-A & C**). Similar spontaneous detrusor activity was also observed when a bladder was distended to 20mmHg and left to accommodate the volume (**Figure 6.4-B & D**). The frequency and amplitude of spontaneous contractions during filling and accommodation were quantified. Spontaneous contractions during filling were measured during the 200s preceding the intravesical pressure reaching 15mmHg. During accommodation activity was measured during the 200s at the end of the 1 hour stabilisation period. Average frequency was represented as contractions per min and average amplitude in mmHg.

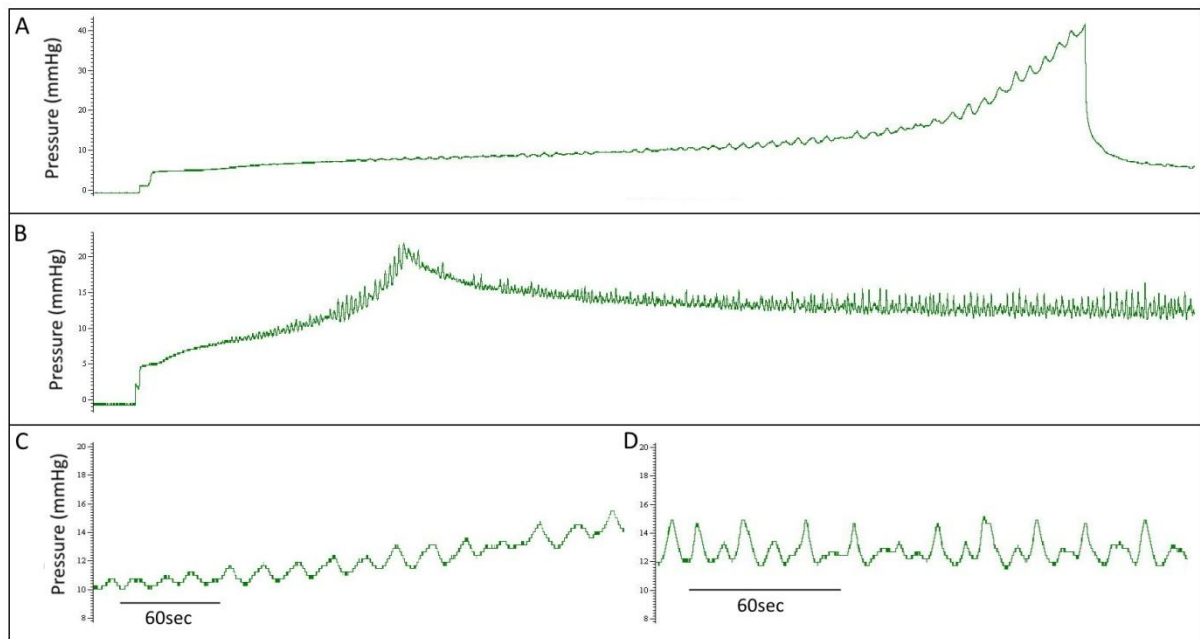


Figure 6.4: Representative traces of a control isolated mouse bladder showing spontaneous phasic contractions during a full distension (A), accommodation from 20mmHg distension (B), and a close up during 200s filling up to 15mmHg (C), a close up during 200s at the end of accommodation (D).

Histology

After experimentation, the whole isolated mouse bladders were fixed in 10% neutral buffered formalin for 24 hours. A catheter attached to a syringe containing formalin was inserted into the bladder through the urethra and secured using silk suture. The bladder was filled with formalin using the syringe and then submerged fully in formalin so that the bladder was fixed in a gently distended state. After 24 hours the bladder was cut in two removing the lower half containing the urethra and keeping the dome of the bladder for processing, sectioning and staining as outline previously (Chapter 2).

6.3 RESULTS

General Effects of Treatment on Animals

Prior to treatment, there was no significant difference in age or weight between animals allocated to each treatment group. After treatment animals in all three groups experienced weight loss. However, animals in the drug treatment groups lost significantly more weight ($3.1\pm0.3\%$ and $3.4\pm0.5\%$ after CPO or IFO treatment respectively) than control animals ($0.8\pm0.4\%$) ($p<0.01$) (**Figure 6.5**). On visual observation, 13 out of 16 CPO treated mice and 12 of 17 IFO treated mice exhibited macroscopic signs of urinary bladder inflammation including redness, increased visible vasculature and areas of haemorrhage (as described in the methods and shown in **Figure 6.2**). CPO or IFO treatment also led to development of considerable oedema in the lamina propria or suburothelial layer resulting in detachment of urothelial cells from the detrusor as can be seen in **Figure 6.6**.

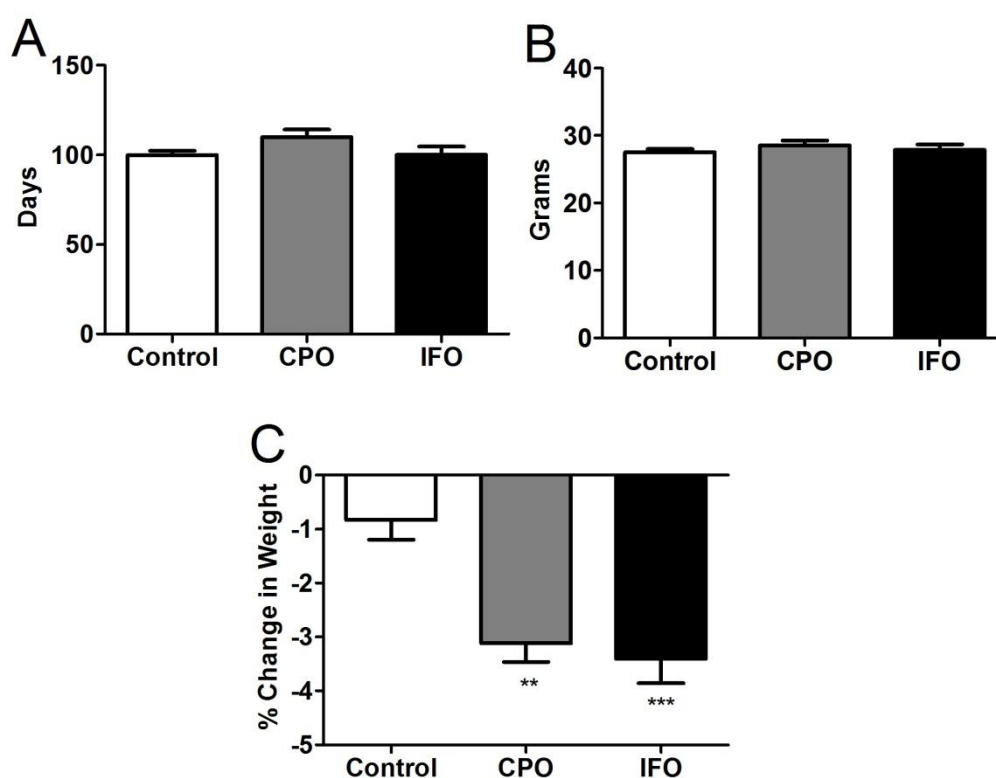


Figure 6.5: Age (A), weight (B) and % change in weight 24 hours after treatment (C) with saline (control), CPO (100mg/kg) and IFO (200mg/kg) in mice. Data represented as mean \pm SEM ($n\geq 8$). Analysed by One-way ANOVA with a Dunnet post-hoc test (** $p<0.01$, *** $p<0.001$ vs control).

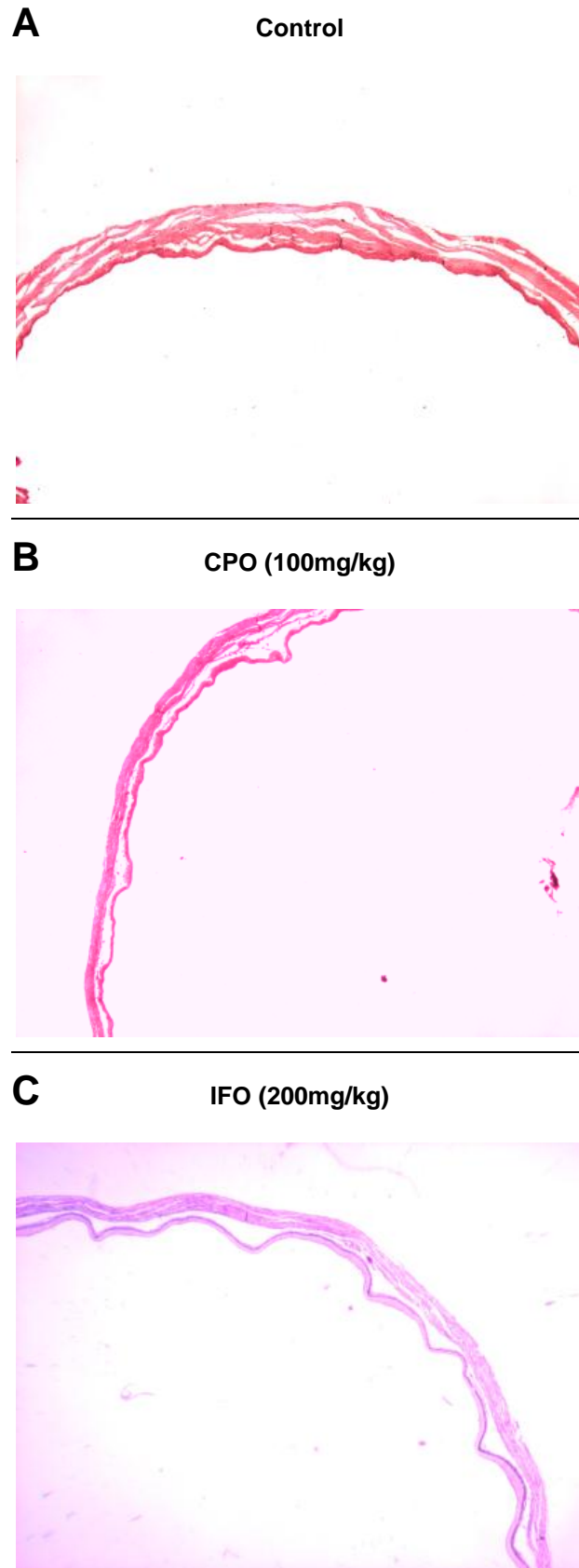


Figure 6.6: Representative images of haematoxylin and eosin staining of isolated mouse bladders treated with saline (control) (A), CPO (100mg/kg) (B) or IFO (200mg/kg) (C). Images representative of n=3 results.

No observable differences were noted in the pressure/volume relationship during distension between the different treatment groups. All treatment groups displayed an initial phase of gradual increase in pressure to 15mmHg followed by a second steep pressure increase beyond that pressure (**Figure 6.7**). This pattern of filling occurred in all bladder groups despite variations in total bladder volume and volumes accommodated in each phase. Average bladder volume was not significantly different between each treatment group ($402\pm 21\mu\text{l}$ in controls, $329\pm 44\mu\text{l}$ in CPO treated and $277\pm 77\mu\text{l}$ in IFO treated mice [$n\geq 5$]).

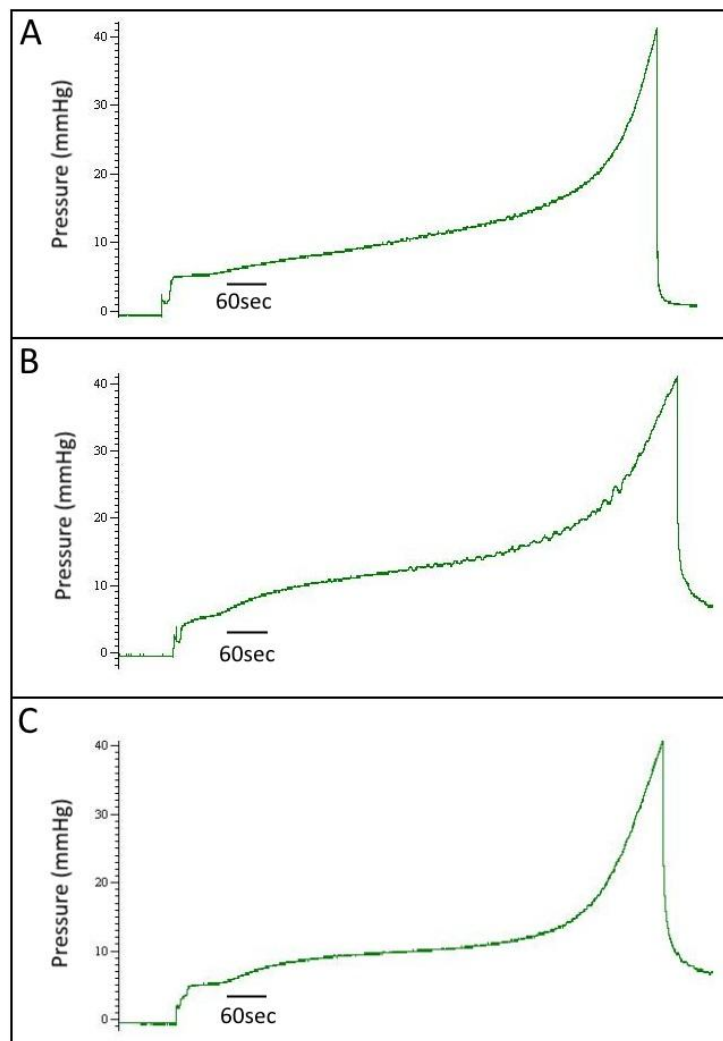


Figure 6.7: Representative traces showing full distension of isolated bladders from saline (control) (A), CPO (100mg/kg) (B), and IFO (200mg/kg) (C) treated mice.

Mediator Release

Samples taken from the lumen of distended bladders and from the serosal solution were analysed for ATP, ACh and PGE₂ content. The concentration of each mediator in each sample was calculated and the luminal and serosal concentrations compared (**Figure 6.8**). Luminal ATP concentrations were 1.5-fold higher than the serosal ATP concentrations ($p<0.05$) (**Figure 6.8-A**). Whereas, luminal concentrations of both ACh and PGE₂ were less than half of the concentrations in serosal samples ($p<0.05$) (**Figure 6.8-B & C** respectively).

However, the volumes of fluid in the lumen and surrounding the serosa of the bladders were different (<0.5mL and 8mL respectively). Therefore, each mediator concentration was then normalised to volume to obtain the total amount present in the total volume of each sample. More than 10 times the amount of ATP (pmoles) was released from the serosa than the lumen ($p<0.001$) (**Figure 6.8-D**). Similarly, the total amount of ACh and PGE₂ was significantly higher (30-fold and 40-fold respectively) in serosal samples than in luminal samples ($p<0.01$) (**Figure 6.8-E & F** respectively).

Three distensions were performed consecutively, with 10 minutes recovery in between, and samples were taken after each to test the repeatability of mediator release in response to stretch. As shown in **Figure 6.9**, total ATP, ACh and PGE₂ release was consistent from both the lumen and serosa of the mouse bladders.

Treatment with CPO (100mg/kg) did not affect the total amount of ATP, ACh or PGE₂ released from either the lumen or the serosa of the mouse bladder (**Figure 6.10**). However, treatment with IFO (200mg/kg) caused a reduction in the luminal ATP levels by 50% ($p<0.05$) and serosal PGE₂ levels by 75% ($p<0.05$). Luminal PGE₂ levels were also reduced by approximately 50% but this was not significant. IFO treatment had no significant effect on ACh levels in luminal or serosal samples.

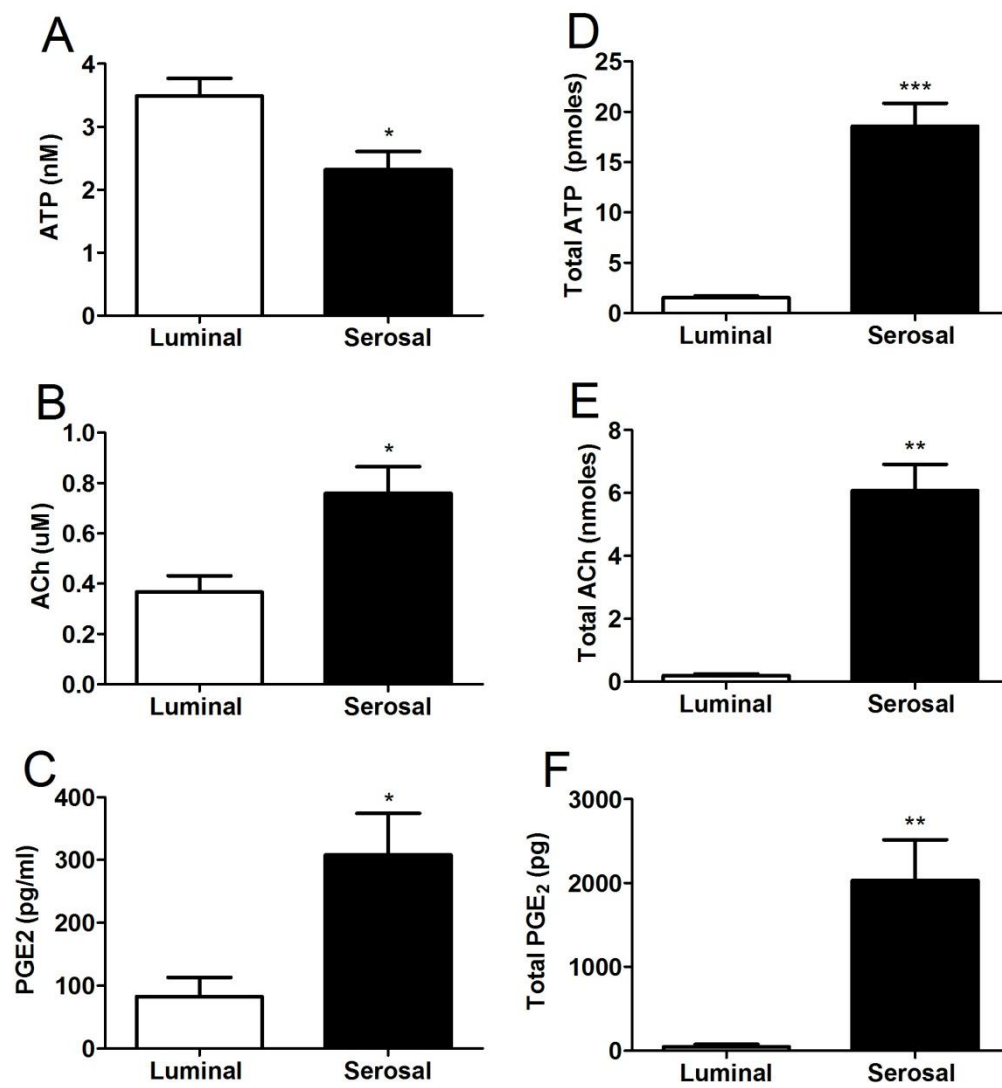


Figure 6.8: ATP, ACh and PGE2 concentrations (A, B & C) and total release (D, E & F) in luminal and serosal samples from isolated mouse bladders. Data represented as mean \pm SEM, n=6. Analysed using a two-way paired Student's t-test (*p<0.05, ***p<0.001 vs control).

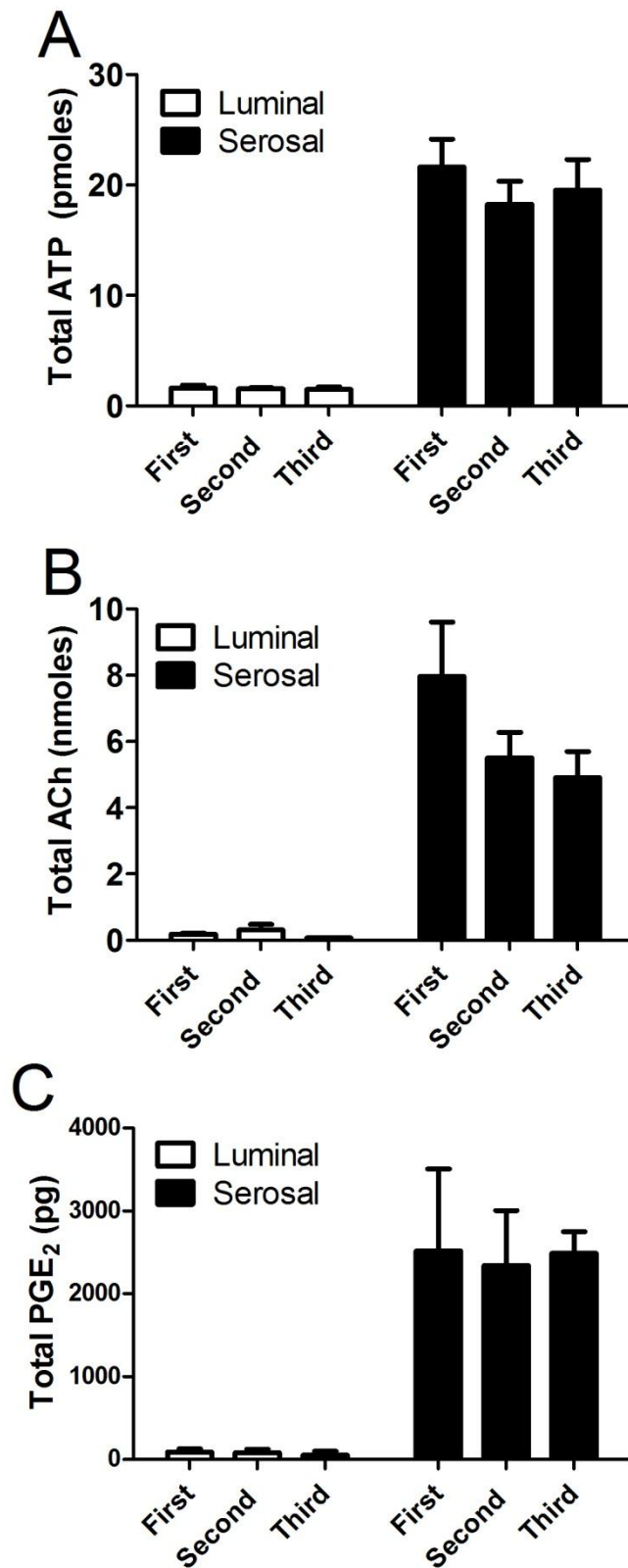


Figure 6.9: Repeatability of total ATP (A), ACh (B) and PGE₂ (C) in three consecutive luminal and serosal samples during stretch of mouse bladders. Data represented as mean \pm SEM (n \geq 3). Analysed via one-way ANOVA with Tukey post-test.

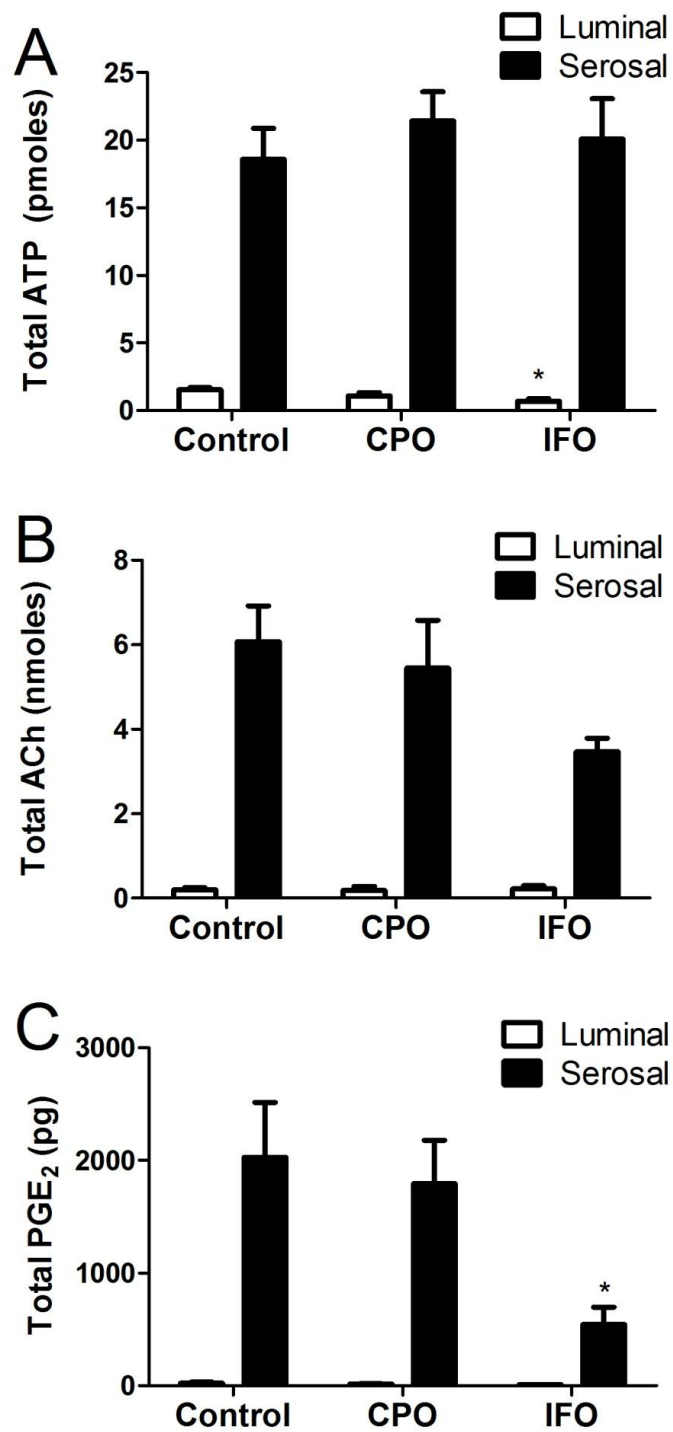


Figure 6.10: Total ATP (A), ACh (B) and PGE₂ (C) in luminal and serosal samples from bladders of mice treated with saline (control), CPO (100mg/kg) or IFO (200mg/kg) at maximal distension. Data represented as mean \pm SEM (n \geq 3). Analysed via one-way ANOVA with Dunnett post-hoc test (*p<0.05 vs control).

Bladder Contractility & Electrical Field Stimulation

Bladders were infused with saline at a rate of 30 μ l/min until intravesical pressure reached 20mmHg at which point infusion was stopped and the outflow tap remained closed. The bladders were then left for approximately 1 hour for the muscle tension to stabilise. All bladders relaxed from their initial pressure of 20mmHg and reached a plateau within an hour (**Figure 6.11**). The volume of the bladders at 20mmHg intravesical pressure was not different between treatment groups (328 \pm 38 μ l in control, 308 \pm 37 μ l in CPO treated and 318 \pm 56 μ l in IFO treated mice respectively [n=6]). There was no difference in compliance of the bladders in each treatment group during filling to 20mmHg or during accommodation of the volume at 20mmHg. In addition, there was no significant difference between the intravesical pressures at plateau of each group (**Figure 6.11**).

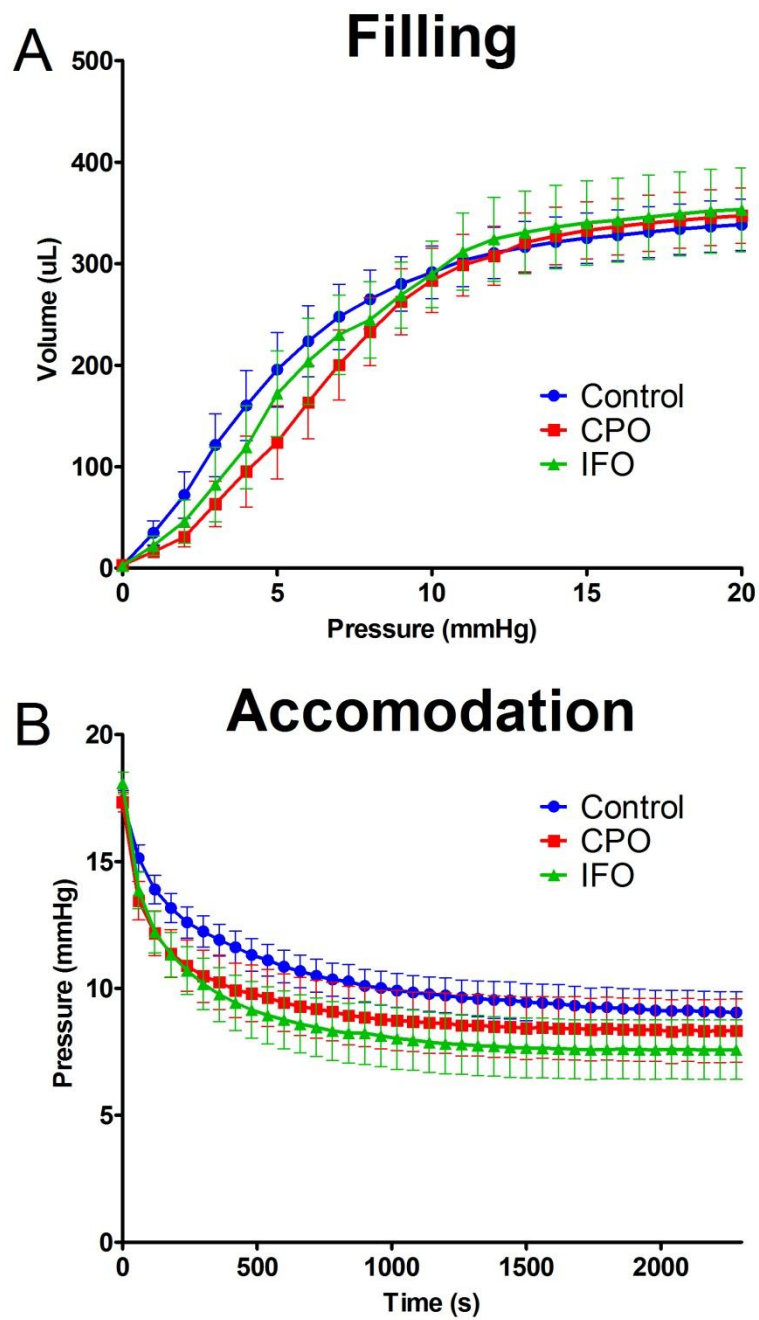


Figure 6.11: Bladder compliance during filling (A) and during accommodation from 20mmHg (B) in mouse bladders treated with saline (control), CPO (100mg/ml) or IFO (200mg/ml). Data represented as mean \pm SEM (n=6). Final time point analysed by one-way ANOVA with a Dunnett post-test.

After intravesical pressure had stabilised, the whole bladder was then electrically stimulated and changes in intravesical pressure recorded. Electrical stimulation of the isolated mouse bladder caused a transient increase in intravesical pressure as shown in **Figure 6.12**. As the frequency of stimulation increased the pressure response also increased (**Figure 6.12-A & B**). The pressure responses at 5, 10 and 20Hz were stable and repeatable (**Figure 6.12-A**) and were completely sensitive to 1 μ M tetrodotoxin (TTx) (**Figure 6.12-C**).

Responses to EFS were reduced by 30-50% in CPO (100mg/kg) or IFO (200mg/kg) treated bladders but this reduction was not significant (**Figure 6.13**). Specifically, at 5Hz responses after CPO or IFO treatment were 4.24 ± 0.53 mmHg and 4.47 ± 2.17 mmHg respectively which was slightly lower than control responses of 8.23 ± 3.17 mmHg. Similar results were seen at 10Hz where responses in CPO or IFO treated bladders were 5.51 ± 0.65 mmHg and 6.98 ± 3.25 mmHg compared to control responses of 11.0 ± 3.10 mmHg. Responses at 20Hz were also a little lower in CPO or IFO treated bladders (8.04 ± 0.91 mmHg and 9.14 ± 3.39 mmHg respectively) than in control bladders (13.4 ± 3.21 mmHg). These differences were not statistically significant and whether this effect was due to any change in efferent nerves or muscle contractility was not apparent.

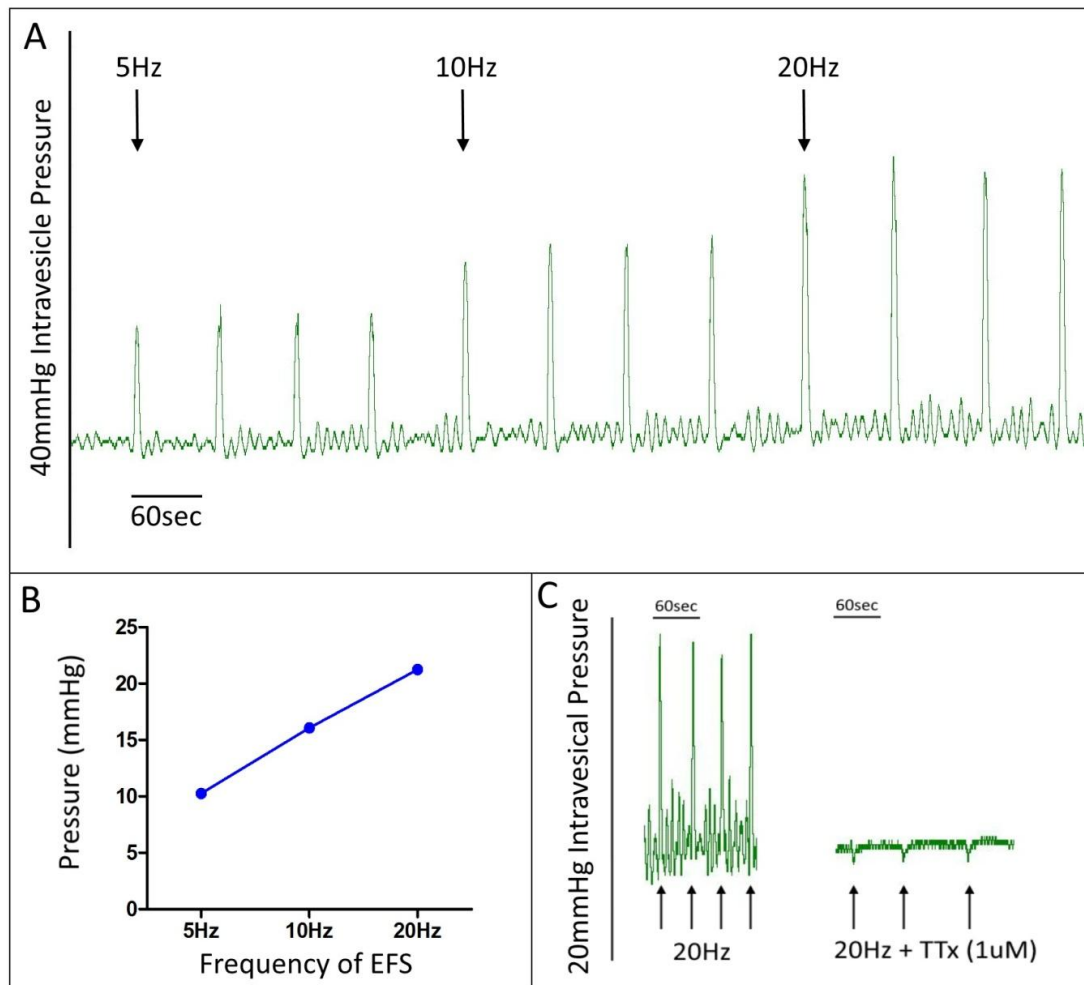


Figure 6.12: Response to electrical field stimulation (EFS) (40 volts and 0.2ms pulse-width, 5 second train every 100s) of a control isolated mouse bladder. A: Representative trace of intravesical pressure increases to EFS at 5, 10 and 20Hz. B: Intravesical pressure response to EFS in an isolated mouse bladder. C: Representative trace of intravesical pressure response to EFS (20Hz) of a mouse bladder in the absence and presence of tetrodotoxin (TTx) (1 μ M).

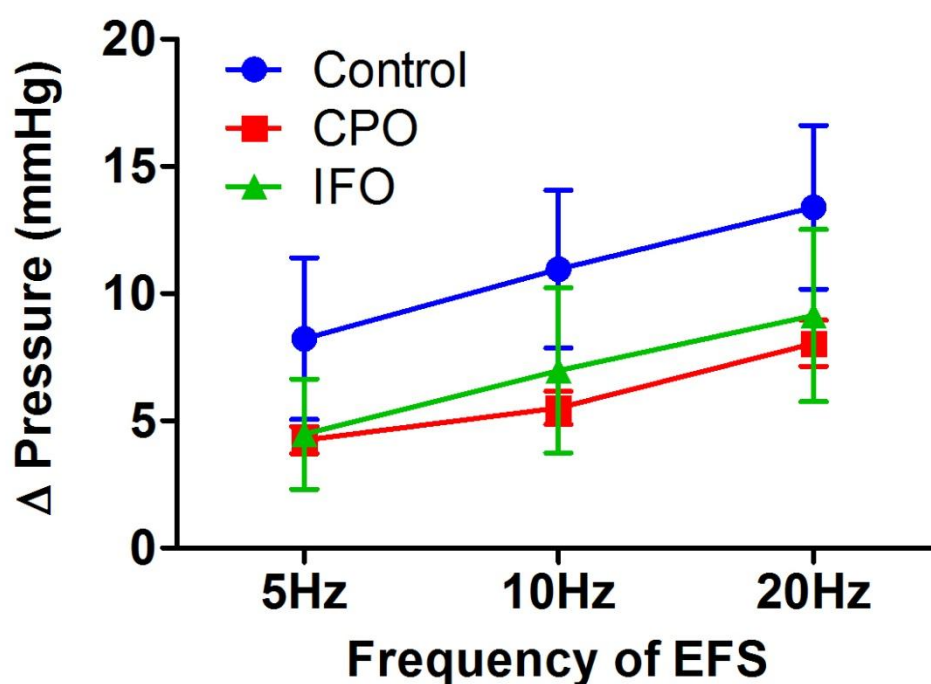


Figure 6.13: Intravesical pressure responses to electrical field stimulation at 5, 10 and 20Hz in isolated mouse bladders treated with saline (control), CPO (100mg/kg) or IFO (200mg/kg). Data represented as mean \pm SEM, n=3 and analysed by two-way ANOVA.

Full frequency curves were performed for each bladder and then responses at 20Hz only were investigated to determine the contribution of NO, ACh and ATP to responses. In control bladders, the baseline response to EFS at 20Hz was 10.3 ± 1.5 mmHg (**Figure 6.14-A**). Removing the effect of NO by adding L-NNA (100μ M) typically caused minor increases in the pressure response (12.3 ± 2.4 mmHg). When the muscarinic contribution to the response was removed after the addition of atropine (1μ M) the responses were similar to baseline (9.0 ± 2.4 mmHg). However, after using $\alpha\beta$ mATP (10μ M) to desensitise the P2X1 purinoceptors, the response to 20Hz EFS was almost eliminated, leaving a response of only 0.7 ± 0.4 mmHg, which was significantly lower than baseline ($p<0.001$).

In mice treated with CPO (100mg/kg) or IFO (200mg/kg) the baseline response to EFS at 20Hz was similar to that in control animals (10.8 ± 2.4 mmHg and 11.3 ± 1.5 mmHg respectively) (**Figure 6.14-B**). CPO or IFO treated mice also exhibited responses similar to control after the addition of L-NNA (100μ M) (10.9 ± 1.4 mmHg and 10.8 ± 1.3 mmHg respectively) and atropine (1μ M) with pressure remaining similar to baseline (7.7 ± 1 mmHg and 7.6 ± 1.3 mmHg respectively). Lastly, after the addition of $\alpha\beta$ mATP (10μ M), only a minor pressure response to 20Hz EFS remained in CPO or IFO treated mice which was again similar to that in control animals (0.8 ± 0.5 mmHg and 1.4 ± 0.5 mmHg respectively).

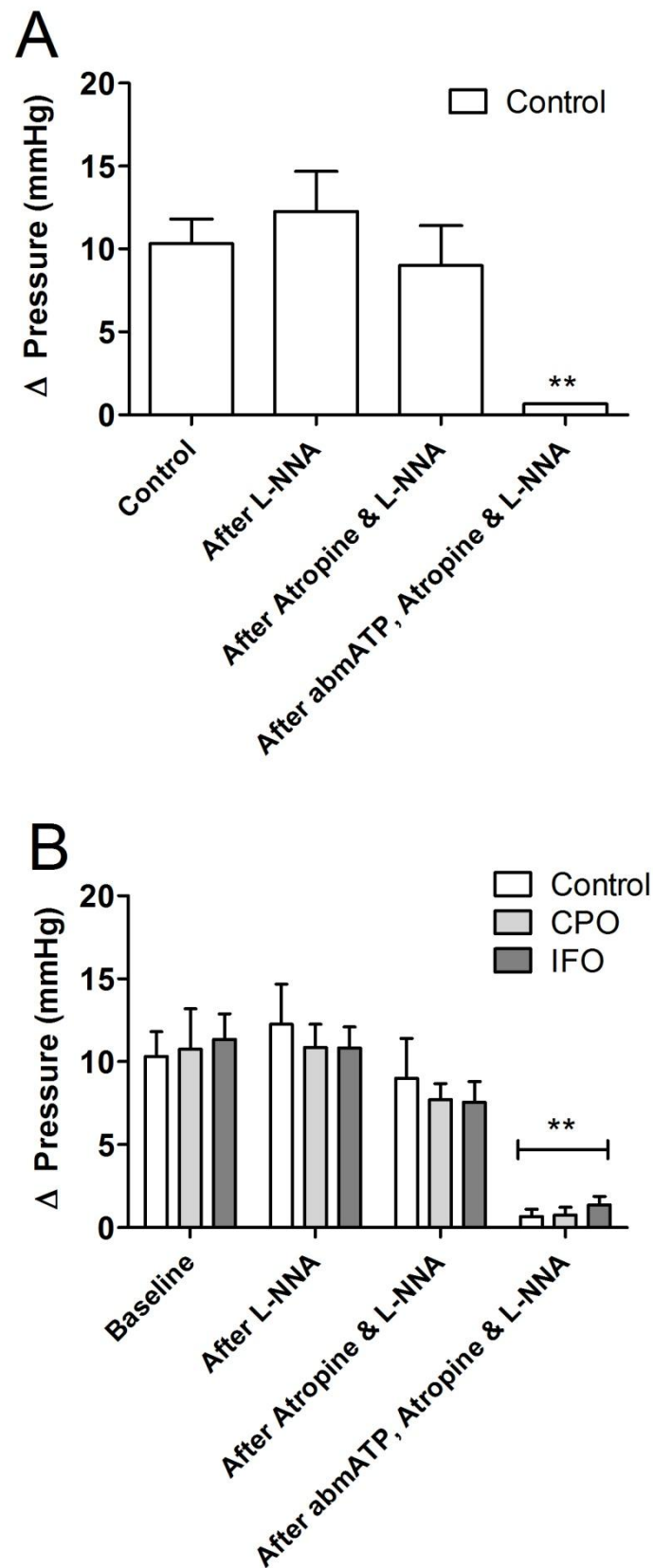


Figure 6.14: Intravesical pressure response to electrical field stimulation at 20Hz at baseline, after L-NNA (100 μ M), atropine (1 μ M) and $\alpha\beta$ mATP (10 μ M) in an isolated mouse bladders treated with saline (control) (A), CPO (100mg/kg) or IFO (200mg/kg) (B). Data represented as mean \pm SEM, $n \geq 4$ and analysed by one-way ANOVA with a Dunnett post-test (** $p < 0.01$ compared to the baseline response of each treatment group).

Responses to Pharmacological Agents

Bladders were infused with saline at a rate of 30 μ l/min to an intravesical pressure of 20mmHg and then left for approximately 1 hour to accommodate the volume and for the muscle tension to stabilise. After plateau, a cumulative dose response curve to isoprenaline (β -adrenoceptor agonist) was performed on each bladder to study the relaxation response (**Figure 6.15**). Bladders treated with CPO (100mg/kg) showed no difference in response to isoprenaline compared to control. However, IFO (200mg/kg) treated bladders had a significantly reduced relaxation response ($p<0.001$) compared to the control response. Mean maximum relaxation responses in the IFO treated bladders were approximately 30% less than controls. The pEC_{50} values for each group were not significantly different (control: 8.1 ± 0.09 , CPO: 8.3 ± 0.09 , IFO: 8.4 ± 0.1).

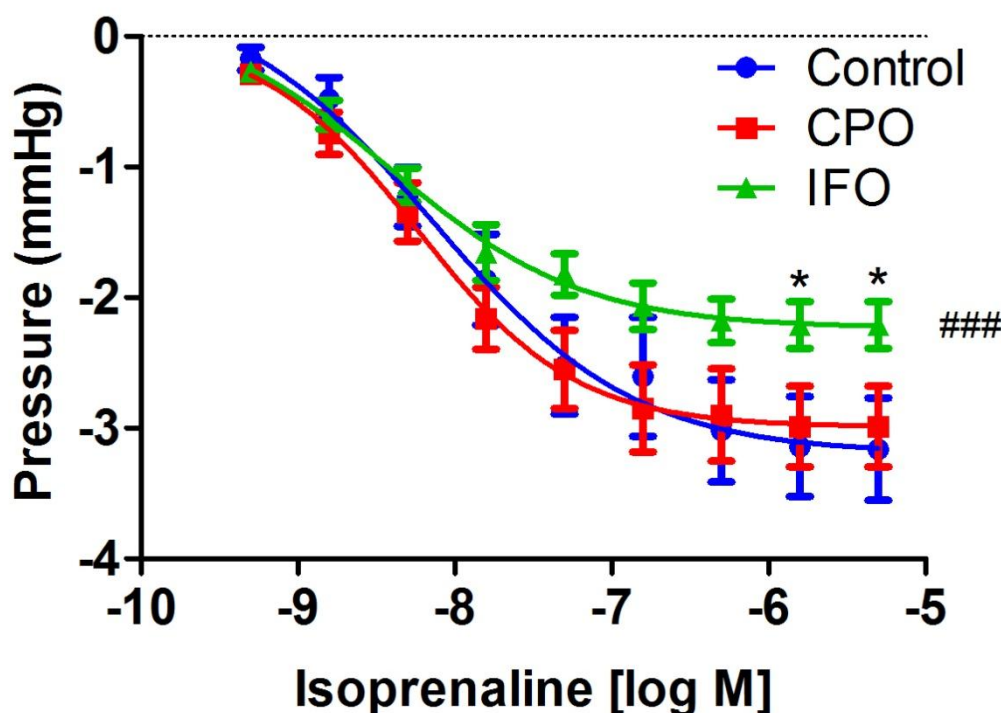


Figure 6.15: Relaxation responses of whole bladders from saline (control), CPO (100mg/kg) and IFO (200mg/kg) pre-treated mice to cumulative doses of isoprenaline. Data represented as mean \pm SEM for each dose ($n\geq 4$). (* $p<0.05$ treated bladder response to isoprenaline vs corresponding control response analysed by one-way ANOVA with Dunnett post-test) (### $p<0.001$ treated curve vs control dose response curve by two-way ANOVA with a Bonferonni post-test).

Spontaneous Contractions

Spontaneous contractions of the isolated bladders during filling and during accommodation were also measured. Treatment with CPO (100mg/kg) or IFO (200mg/kg) had no significant effect on the average frequency (**Figure 6.16-A**) or the amplitude (**Figure 6.16-B**) of spontaneous contractions compared to control.

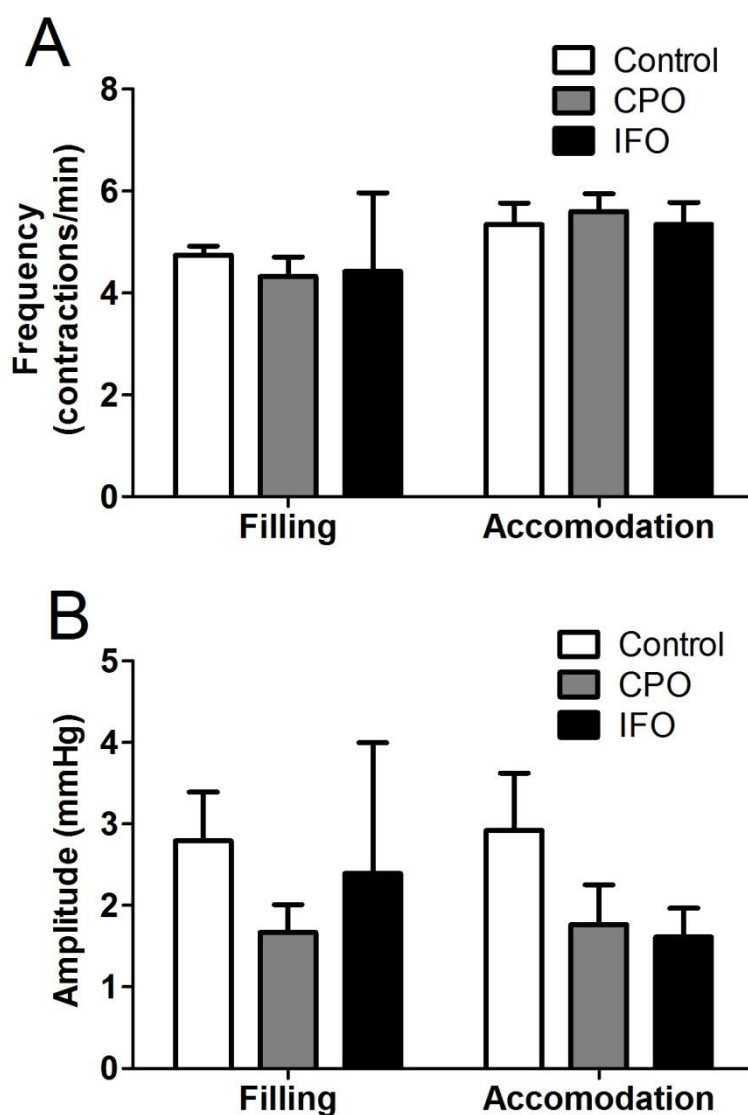


Figure 6.16: Average frequency (A) and amplitude (B) of spontaneous contractions in bladders from saline (control), CPO (100mg/kg) and IFO (200mg/kg) treated mice during filling to 40mmHg and accommodation at 20mmHg pressure. Data represented as mean \pm SEM (n \geq 24). Analysed by one-way ANOVA with Tukey post-test.

6.4 DISCUSSION

The activity of afferent nerves in the bladder can be influenced by various factors including urothelial mediators, detrusor tone as well as the sensitivity of the nerves themselves (de Groat and Yoshimura, 2009). The previous Chapter demonstrated an increase in afferent activity during distension in CPO and IFO treated bladders. Accordingly, the present study aimed to investigate whether urothelial function or muscle contractility changes after treatment with these drugs could be contributing to the increase in afferent activity.

Increasing age has also been shown to be associated with increased afferent excitability in humans and mice (Gregorini et al., 2015, Daly et al., 2014). In the present study there was no difference in the age or weight of the animals before treatment. However, CPO and IFO treatment caused the animals to lose weight over the following 24 hours. Lower body weights of animals treated with CPO have been reported previously and therefore this was not an unexpected result (Mok et al., 2000, Boudes et al., 2011). Given that initial weight, age and bladder volume did not differ between the groups any differences in tissue responses was assumed to be due to treatment with CPO or IFO.

Urothelial Effects of CPO and IFO

Treatment with either CPO or IFO caused macroscopic signs of bladder inflammation including redness, increased visible vasculature and areas of haemorrhage. Treatment with these drugs also caused substantial swelling and oedema in the lamina propria layer resulting in detachment of the urothelial layer from the detrusor. The effects appeared to be slightly more severe after IFO treatment. It should be noted that these effects were observed subjectively and were not quantified. However, similar inflammatory effects have been observed in rats treated with CPO or IFO (Macedo et al., 2011, Juszczak et al., 2010, Macedo et al., 2008a, Giglio et al., 2007, Giglio et al., 2005). Specifically, acute treatment with CPO caused bladder redness, oedema, urothelial erosion and ulceration in rats (Juszczak et al., 2010). Swelling of the lamina propria sufficient to cause detachment of urothelial cells from the detrusor has also been reported in mice treated with CPO (Golubeva et al., 2014). Similarly, after 24 hours, IFO treatment in rats induced severe haemorrhagic cystitis showing marked oedema, intense urothelial damage and loss of folding, leukocyte infiltration, and haemorrhage (Macedo et al., 2008a, Macedo et al., 2011). Similar haemorrhage, oedema, urothelial ulceration and inflammatory cell infiltration has also been reported up to 24 hours after intravesical instillation of acrolein in rats and mice. (Macedo et al., 2008b, Batista et al., 2006, Wang et al., 2008b). This confirms that the CPO

and IFO treatments used in the present study effectively caused cystitis consistent with previous studies.

As discussed previously, after CPO treatment in rats the urothelium undergoes an acute period of damage characterised by ulceration and erosion (Auge et al., 2013, Juszczak et al., 2010) followed by a period of rapid proliferation which results in urothelial hyperplasia (Boudes et al., 2011, Golubeva et al., 2014, Romih et al., 2001). Approximately a week after the initial CPO treatment, the hyperplastic urothelium starts differentiating and apoptosis increases to remove cells until a normally differentiated three-layered urothelium is restored by day 14. Chronic CPO treatment (four injections over seven days) in mice has been shown to cause urothelial hyperplasia due to an increased number of cells (Boudes et al., 2011, Golubeva et al., 2014). Typical signs of oedema, swelling and leukocyte infiltration were also present, but no signs of urothelial erosion or ulceration were observed after chronic CPO treatment. Loss of urothelial cells or hyperplasia during the early and late stages of CPO or IFO induced damage could alter the levels of mediators released by the urothelium and affect the activity of afferent nerves. In particular, the release of ATP and PGE₂ from the urothelium has been linked to bladder hyperactivity due to the possible activation or sensitisation of sensory nerves (de Groat, 2004).

The present study demonstrated release of ATP, ACh and PGE₂ into the lumen of the bladder as well as from the serosal side of the bladder during stretch. Mediator levels in luminal and serosal samples from control bladders were consistent and repeatable. Interestingly, the amount of each mediator in the serosal samples was greater than the amount in the luminal samples. This supports previous findings in rabbit urinary bladder showing that ATP is released from the baso-lateral surface of the urothelium during stretch rather than the apical/luminal surface (Dunning-Davies et al., 2013, Ferguson et al., 1997). More specifically, Ferguson et al., (1997) demonstrated basal ATP release from both luminal and serosal surfaces of the rabbit bladder, however stretch induced an increase in ATP release from the serosal side only. They also demonstrated that the source of this ATP was the urothelium and not the muscle. Similarly, Dunning-Davies et al., (2013) showed that distension of isolated rabbit urothelium caused an increase in ATP release from the serosal surface. This is understandable given that the target tissues of urothelial mediators are under the urothelium and not in the lumen. Furthermore, the surface of the urothelium is a barrier that prevents entry of molecules into the bladder wall from the lumen and therefore it is logical that it also prevents movement of molecules in the opposite direction.

Treatment with CPO did not affect the levels of ATP, ACh or PGE₂ in luminal or serosal samples from the mouse bladder. However IFO treatment caused a reduction in luminal ATP levels and serosal PGE₂ levels but did not affect ACh levels. This is unexpected given that Smith et al., (2005) have previously reported increased ATP release from rat urothelium after CPO treatment. However, the model used a 50% higher dose of CPO administered chronically every three days for a total of three treatments. Therefore, the increased ATP release seen by Smith et al., (2005) may be due to the presence of a hyperplastic urothelium with more cells releasing ATP or may be a result of greater urothelial damage allowing ATP leakage into the lumen. In the present study, urothelial damage appeared to be greater after IFO treatment. This damage may have caused more death or loss of urothelial cells resulting in a decreased number of cells releasing ATP and therefore lower luminal ATP levels. No studies have reported on the luminal release of PGE₂ after CPO or IFO treatment despite several studies in rodents demonstrating an increased expression of COX-2 in urothelial cells 12-24 hours after treatment with either drug (Macedo et al., 2011, Macedo et al., 2008a, Klinger et al., 2007). One study using a very high dose of IFO (400mg/kg) in rats found that the plasma PGE₂ concentration was increased 24 hours after treatment (Macedo et al., 2011) however the exact source of this PGE₂ in the bladder is not clear. Again, the present study demonstrated a decreased serosal level of PGE₂ after IFO treatment. This may be due to detachment of the urothelium from the detrusor making it more difficult for PGE₂ to be released from the serosa. However, luminal PGE₂ levels also tended to decrease after IFO treatment suggesting that a loss of urothelial cells may be responsible for the lower levels. Urothelial damage after CPO or IFO treatment would be expected to disrupt the barrier function allowing mediators to move into the lumen resulting in higher levels of all mediators in the luminal samples. This was not observed in the present study suggesting that the barrier function was not compromised or that urothelial cell loss was sufficient to result in overall lower levels in the lumen.

In any event, the increased afferent activity seen after treatment with CPO or IFO in the previous Chapter does not appear to be due to increased ATP or PGE₂ release from the urothelium. Therefore, the acute stage of CPO or IFO bladder hyperactivity may involve a non-urothelial mechanism of nerve sensitisation. The urothelial hyperplasia reported in other studies appeared several days after CPO administration or during chronic CPO administration. This suggests that increased mediator levels may appear due to more numerous urothelial cells at this later stage when afferent nerves are already sensitised, resulting in further enhancement of afferent activity.

Muscle Effects of CPO and IFO

The detrusor smooth muscle has myogenic tone and modulation of this tone may influence the ability of the bladder to accommodate urine during filling (Andersson, 1999). Furthermore, enhanced muscle tone during filling may also cause enhanced afferent nerve activity resulting in abnormal sensations or dysfunction. Bladder muscle tone can be influenced by a number of different mechanisms including urothelial transmitter release, receptor expression, altered efferent or afferent nerve activity, altered excitability or coupling of smooth muscle cells and ICs or changes to spontaneous contractile activity (Fry et al., 2010, Andersson, 2004).

As discussed previously, rodents treated with CPO or IFO demonstrate enhanced intravesical pressure during filling, reduced bladder capacity, increased urinary frequency as well as decreased contractile force during voiding (Okinami et al., 2014, Macedo et al., 2011, Boudes et al., 2011, Everaerts et al., 2010b, Wang et al., 2008b, Kageyama et al., 2008, Juszczak et al., 2007, Hu et al., 2003). Basal pressure of the bladder has also been shown to be increased in rodents treated with these drugs (Boudes et al., 2011, Macedo et al., 2011, Juszczak et al., 2007). Furthermore, in conscious rats, CPO treatment (200mg/kg) has also been shown to enhance the spontaneous activity of the bladder, particularly during the filling phase (Pan et al., 2012). Isolated bladder strips from CPO treated mice also showed enhanced spontaneous contractile activity after application of an L-type Ca^{2+} channel activator (Okinami et al., 2014). Accordingly, it appears that both CPO and IFO affect detrusor tone leading to storage symptoms. In the present study, treatment with CPO or IFO did not affect compliance of the isolated mouse bladder during filling or during accommodation as the respective pressure-volume curves were almost identical between each group. In addition, the frequency and amplitude of spontaneous contractions were unchanged after CPO or IFO treatment. However, the present study used an isolated bladder technique whereas the storage symptoms reported in rodents were in anaesthetised or awake animals with intact nervous systems. Accordingly, it appears that enhanced bladder tone due to CPO or IFO treatment is dependent on the presence of efferent or afferent nerve activity.

In addition, the ability of the detrusor to relax in response to β -AR stimulation may also be altered manifesting as an increase in tone. Therefore, the effect of CPO or IFO on the bladder relaxation to the β -AR agonist isoprenaline was investigated. The distended bladder relaxed in response to isoprenaline and the relaxation was concentration dependent. CPO treatment did not affect the ability of the bladder to relax to isoprenaline whereas IFO treatment inhibited the relaxation response. Previous studies have reported that 3 days after CPO treatment

(100mg/kg) isolated rat bladder strips had inhibited relaxations to isoprenaline (Giglio et al., 2007, Vesela et al., 2012). The results in the present study in mice used the same dose of CPO, but looked at effects 24 hours after treatment. The responses seen after 24 hours do not support the previous findings in rats suggesting these effects either do not occur in mice or develop at a later stage of CPO induced cystitis. However, IFO treatment (200mg/kg) did affect isoprenaline relaxations suggesting that IFO induced cystitis causes similar changes to CPO in rats. However, Giglio et al., (2007) found that there was no postjunctional β -AR mediated relaxation in response to electrical field stimulation in control or CPO treated bladder strips. Therefore, although CPO or IFO treated bladder tissue may have a reduced ability to relax to β -AR stimulation, this mechanism does not appear to play a role in nerve evoked responses and is unlikely to be responsible for the storage symptoms seen in animals after treatment.

Therefore, the question is whether there are altered excitatory nerve responses that may contribute to altered detrusor tone. In whole mouse bladders, stimulation of the efferent nerves by electrical field stimulation produced repeatable, transient, TTx sensitive and frequency dependent contractions. This is similar to a previous study using EFS (50V, 0.2ms pulse width, 3 second train) on whole mouse bladders (Fabiyyi and Brading, 2006) and responses in whole bladders were found to be comparable to isolated bladder strips. In the present study, blocking NO production with L-NNA had no effect on EFS induced responses, although an increased trend was apparent. Atropine did not significantly reduce responses, while $\alpha\beta$ mATP almost completely abolished EFS induced contractions. These results suggest that ATP makes up the large majority of the EFS induced contraction in whole mouse bladder and that NO and ACh potentially contribute to a small relaxation and contraction response respectively.

These results do not fully agree with previous findings in whole bladders or isolated bladder strips from mice where atropine and $\alpha\beta$ mATP reduced EFS (20Hz) induced contractions by approximately 50% each, suggesting ACh and ATP contribute equally to nerve induced contractions in the mouse bladder (Fabiyyi and Brading, 2006, Mok et al., 2000). Similar results have been reported for isolated bladder strips from rats with atropine causing almost 50% reduction of the EFS induced contraction and $\alpha\beta$ mATP almost eliminating the remaining response (Giglio et al., 2007). NO has been reported to have both excitatory and inhibitory effects on detrusor muscle and therefore, the influence of NO in the bladder is complex (Meng et al., 2012, Yanai et al., 2008). Specifically, it appears that a cGMP-dependent pathway causes relaxation of the detrusor smooth muscle while a cGMP-independent pathway involving Ca^{2+} release increases spontaneous activity of the detrusor (Yanai et al., 2008). However, blocking

NO production with L-NNA has previously been shown to have no effect on EFS induced responses in healthy rat bladder strips (Vesela et al., 2012).

Bladders from mice treated with CPO (100mg/kg) or IFO (200mg/kg) showed no significant difference in responses to EFS compared to control bladders although a general decrease in contraction was noted. These results support previous findings that show no difference in EFS induced responses during the acute stage (<1 day) of CPO induced cystitis, but a reduction in responses at a later stage (3 days) in rodents. Specifically, the acute effects of CPO (200mg/kg) induced cystitis were investigated in isolated bladder strips from shrew and there was no change in the frequency response curves (1-16Hz) compared to controls (Mok et al., 2000). Two studies have used isolated bladder strips from rats treated with CPO (100mg/kg) three days earlier and found that responses to EFS were approximately half those of control responses (Giglio et al., 2007, Vesela et al., 2012). However, general contractility to KCl was affected to a similar extent as the EFS induced contractions suggesting CPO treatment affects muscle function rather than efferent nerve function.

The results of the current study also demonstrated no difference in contribution of NO, ACh or ATP to the EFS induced responses in CPO or IFO treated bladders compared to controls. These results support findings from both acute and late stage models wherein atropine and $\alpha\beta$ mATP reduced responses to a similar extent in control and CPO treated bladder strips (Giglio et al., 2007, Mok et al., 2000). However, in the late stage model, inhibition of NO production enhanced EFS induced contractions in CPO treated bladder strips but not in control strips (Vesela et al., 2012). Previous studies have suggested that muscarinic receptor stimulation on the urothelium causes enhanced NO release reducing the muscarinic detrusor contraction in CPO treated rats (Andersson et al., 2008). Therefore this mechanism may be responsible for the increased nerve mediated contraction seen by Vesela et al., (2012) after blocking NO production. However, these effects were observed during the later stage of CPO induced cystitis and the present study suggests that enhanced NO production does not contribute to EFS induced responses during the acute (24 hour) stage of CPO or IFO induced urotoxicity.

Therefore the results of the present study support previous findings that, 24 hours after CPO treatment, the efferent nerve mediated bladder responses are not significantly different from controls. Responses in IFO treated bladders were similar to CPO treated bladders. However, treatment with either CPO or IFO did cause a slight reduction in contraction to EFS supporting a transition to reduced muscle contractility at a later stage of cystitis.

Implications for Afferent Nerve Activity and Bladder Hyperactivity

As mentioned earlier, the activity of afferent nerves in the bladder can be influenced by various factors including urothelial mediators, detrusor tone as well as the sensitivity of the nerves themselves (de Groat and Yoshimura, 2009). The results of the present study have demonstrated that CPO or IFO treatment, despite causing an increase in afferent activity during distension, does not cause an increase in stimulatory urothelial mediator release and does not affect bladder muscle tone or contractility. Therefore, it appears that CPO or IFO treatment leads to enhanced sensitivity of the afferent nerves themselves resulting in enhanced activity during filling. This also supports the previous findings of increased action potentials in response to current injection in isolated DRG neurones from CPO treated rats (Yoshimura and de Groat, 1999, Dang et al., 2008).

In the present study, there was no increase in detrusor tone during filling of bladders treated with CPO or IFO despite *in vivo* studies reporting reduced bladder compliance and increased basal pressure leading to decreased bladder capacity and increased micturition frequency. Similarly, in the previous Chapter, no compliance changes were observed despite there being an increase in afferent firing during filling. In the nerve recording preparation, the afferent nerves were cut in order to record afferent firing in an electrode, thereby removing any effect of spinal reflexes on the results. Similarly, the whole bladder preparation requires isolation of the bladder thereby removing any afferent reflexes. Therefore, it is hypothesised that the changes to detrusor tone are due to enhanced afferent activity causing reflex activation of a tonic efferent pathway.

Afferent and efferent responses are difficult to investigate simultaneously *in vitro* however a new *in situ* technique in rats has been developed in which the bladder is dissected along the midline from dome to urethra to create two separate parts while maintaining the blood vessels, pelvic nerves and spinal reflexes intact (Aronsson et al., 2013). This preparation enables investigation of reflex effects by applying mechanical or electrical stimuli to one side of the bladder or its afferent nerves while measuring the response from the other side. Using this technique, Aronsson et al., (2014) then examined the effect of CPO pre-treatment on the responses of the rat bladder. Responses to electrical stimulation of the efferent pelvic nerves were reduced in the CPO treated rats. However after CPO treatment, mechanical stretch of one side of the bladder elicited significantly larger contractions of the contralateral side compared to control responses. This supports the hypothesis that enhanced bladder tone after CPO or IFO treatment is due to enhanced afferent activity causing reflex activation of an efferent tonic effect.

The reflex induced responses almost instantaneously disappeared after removal of the stretch force in control bladders, but the responses in CPO treated bladders demonstrated a gradual decline and spontaneous contractions persisted for several minutes (Aronsson et al., 2014). Accordingly, it appears that enhanced spontaneous activity seen after CPO or IFO treatment could also be due to enhanced afferent reflex activity in response to stretch. Furthermore, electrical stimulation of the afferent pelvic nerve produced similar responses in control and CPO treated bladders suggesting that the sensitisation may be occurring at the mechanoreceptor level. The results in the previous Chapter support this finding as afferent activity was increased during filling in response to bladder distension presumably activating mechanoreceptors.

Conclusions

CPO and IFO treatment did not increase the release of stimulatory urothelial mediators ATP or PGE₂ from whole mouse bladders during stretch from either the luminal or serosal side of the bladder. In addition, detrusor compliance during filling or accommodation was not altered after CPO or IFO treatment. Similarly, the bladder response to EFS was similar in controls and treated bladders. Therefore, the enhanced total nerve activity in the mouse bladder during graded distensions observed in the previous Chapter appears to be due to direct sensitisation of the nerves themselves and not as a result of altered urothelial or detrusor function. These nerve results are supported by previous findings and are most likely due to sensitisation of the mechanoreceptors resulting in enhanced activity at equivalent bladder volumes. These findings potentially explain the pain, frequency and abnormal sensations experienced by patients after treatment with CPO or IFO.

CHAPTER 7: General Discussion

The aim of this thesis was to increase our understanding of the bladder toxicity caused by cyclophosphamide and ifosfamide and the implications of this toxicity on bladder function. Accordingly, a body of work is described investigating the effects of cyclophosphamide, ifosfamide and their toxic metabolites on the function of the urothelium, detrusor and the afferent and efferent nerves in the bladder. A variety of techniques and tissue types were used to investigate how these substances affect the function of isolated parts of the bladder as well as the function of the bladder as a whole. Therefore the final discussion focuses on the interaction between the tissue types and how each may contribute to overall bladder function.

As discussed throughout this thesis, CPO and IFO are cytotoxic drugs used extensively in the treatment of cancer and autoimmune diseases (Furlanut and Franceschi, 2003, Brode and Cooke, 2008). A major limiting factor in their use is the resulting bladder toxicity which can cause both anatomical damage and bladder function changes. The metabolites of CPO and IFO come into direct contact with the urothelium causing urothelial damage followed by severe bladder inflammation (Cox, 1979, Lima et al., 2007). The functional effects can manifest as bladder pain, urinary frequency, urgency, dysuria, and feelings of incomplete emptying (Korkmaz et al., 2007, Fukuoka et al., 1991) and these effects can last beyond the resolution of inflammation and damage. These lasting effects significantly affect the quality of life of patients both during chemotherapy and after treatment. The current protective measures have limited efficacy and a significant percentage of patients treated with CPO or IFO are still likely to suffer from urotoxic side effects of these drugs. Understanding how these drugs cause bladder damage and what functional changes occur as a result may reveal future targets for preventing the adverse effects of these drugs or restoring normal bladder function after treatment.

Inflammation has been shown to increase bladder afferent neurone excitability and is thought to contribute to bladder overactivity and pain (Hayashi et al., 2009, Wyndaele and De Wachter, 2003, Yoshimura et al., 2002). Accordingly, the inflammation during CPO or IFO induced cystitis may be intimately linked to the long term changes in bladder function. Patients with painful bladder syndrome (PBS) exhibit very similar urinary symptoms to those with CPO or IFO induced cystitis including urinary frequency, urgency and pain (Parsons, 2007) and evidence suggests these symptoms may be a result of bladder inflammation (Amaravadi et al., 2011, Erickson et al., 2002, Keay, 2008, Erickson et al., 2008, Wyndaele et al., 2009, Ogawa et al., 2010, Peters et al., 1999). The results in this thesis, in combination with previous findings, support the concept that inflammation may have a role in the bladder dysfunction seen after CPO or IFO induced cystitis. Therefore, the following discussion will address whether inflammation may play a role in the bladder symptoms occurring after CPO or IFO treatment.

Sources of Damage and Inflammation in CPO or IFO Induced Cystitis

Increased Urothelial Permeability

After CPO treatment the urothelium undergoes an acute period of damage characterised by ulceration and erosion resulting in the loss of the epithelial barrier function (Auge et al., 2013, Juszcak et al., 2010). The permeability barrier of the urothelium is compromised in various urinary bladder disorders such as PBS and overactive bladder (Hauser et al., 2008, Hurst et al., 1996, Parsons, 2007, Parsons, 2011, Teichman and Moldwin, 2007). Specifically, in patients with PBS a loss of urothelial barrier function was demonstrated by an increase in urea movement across the urothelium (Parsons et al., 1991). However, in these patients it is not clear whether the loss of barrier function causes the associated inflammation or is a secondary effect of inflammation. A recent study in rats used intravesical instillation of protamine sulphate to disrupt the urothelial barrier function and found that inflammation resulted (Soler et al., 2008). However, in rats where urine production was inhibited no inflammation occurred. Accordingly, it appears that disruption of the urothelial barrier itself does not cause inflammation, but allows noxious substances in the urine to move into the bladder and cause inflammation. This may explain the mechanism of toxicity of CPO or IFO in the bladder whereby acrolein and/or CAA causes damage to the urothelium resulting in loss of the barrier function and allowing further penetration of the metabolites and other urinary irritants into the bladder wall causing or exacerbating inflammation.

The results in this thesis also support disruption of the barrier function of the urothelium. Specifically when acrolein or CAA was applied to the luminal surface of porcine bladder, not only was the urothelium damaged, but the contractility of the detrusor tended to be reduced. This suggests that both metabolites were capable of causing sufficient damage to the urothelium to allow penetration into the detrusor where they affected muscle responsiveness. Given that this damage occurred after only a four hour incubation it appears likely that acrolein or CAA can cause direct disruption of the urothelial barrier function and detrusor damage prior to the development of inflammation. Accordingly, inflammation may be a result of tissue damage after infiltration of the toxic metabolites and may worsen the direct damage caused by the metabolites. Although excretion of the toxic metabolites is complete by 24 hours post-treatment, the peak in inflammation and the greatest effects on contractile responses have been shown to occur at 60 hours post-treatment (Giglio et al., 2005, Vesela et al., 2012). Accordingly, this suggests the metabolites are responsible for urothelial damage and initiating inflammation which is then responsible for changes to detrusor responsiveness.

Urothelial Release of Inflammatory Mediators

Acrolein or CAA treatment was demonstrated to cause cultured human urothelial cells to release more ATP and PGE₂ during basal and stretch conditions (Chapter 3). These two mediators can have a proinflammatory effect in the bladder. Save & Persson (2010) demonstrated that human urothelial cells released IL-8 in response to P2Y receptor stimulation. Furthermore, they demonstrated that urothelial cells infected with *Escherichia coli* had enhanced extracellular ATP and IL-8 levels and that the increased IL-8 could be reduced by increasing breakdown of the endogenous ATP with apyrase. This suggests that enhanced ATP from the urothelial cells can act in an autocrine manner to stimulate release of cytokines such as IL-8. PGE₂ release from urothelial cells has also been shown to have a proinflammatory effect and blocking EP4 receptors during CPO induced cystitis caused a reduction in inflammation and inflammatory cell infiltration (Chuang et al., 2012).

Urothelial cells have also been shown to release various cytokines, including IL-1 β , IL-6 and IL-8 in response to infection or cytotoxic damage which have various inflammatory effects in the bladder (Funfstuck et al., 2001, Kang et al., 2013). The effects of these cytokines include actions such as recruitment of inflammatory cells, induction of phagocytic activity, cellular proliferation and angiogenesis. Furthermore, oxidative stress has been shown to cause increased IL-8 production in intestinal epithelial cells (Yamamoto et al., 2003). Therefore, given that acrolein caused increased oxidative stress and urothelial cell damage it is not surprising that it also caused an increase in IL-1 β , IL-6 and IL-8 release in the present study. Additionally, the increased ATP release from the urothelial cells may also have caused increased cytokine release, in particular IL-8. Therefore, the urothelial cells themselves may be the initial mediators of the inflammatory response to CPO and IFO induced cystitis due to acrolein or CAA enhancing the release of mediators such as ATP, PGE₂ and cytokines.

Neurogenic Inflammation

Neurogenic inflammation includes both vascular and non-vascular inflammatory effects triggered by the release of proinflammatory neuropeptides from sensory nerve terminals in response to noxious stimuli (Geppetti et al., 2008). The inflammatory neuropeptides in sensory nerve terminals include substance P, CGRP and neurokinin A and they can cause arteriolar vasodilation, plasma extravasation and oedema as well as alterations in smooth muscle activity (Morrison, 1999, Geppetti et al., 2008).

The release of neuropeptides from sensory nerves has been associated with the activation of various receptors and channels on the nerves including α 1-ARs, TRPV1 and TRPA1 (Geppetti

et al., 2008). Phenylephrine caused release of neuropeptides from sensory nerves and subsequent plasma extravasation in the bladder of the rat (Trevisani et al., 2007a). Similarly, activation of TRPV1 or TRPA1 channels has also been shown to cause neurogenic inflammation in guinea pig lung and rat oesophagus respectively (Trevisani et al., 2005, Trevisani et al., 2007b).

TRPV1 channels are expressed on sensory neurones in pain pathways and their activation by various stimuli (such as capsaicin) causes sensations of pain or discomfort (Rosenbaum and Simon, 2007). TRPA1 channels are coexpressed with TRPV1 in sensory nerves supporting their role in the nociception pathway (Bautista et al., 2005). TRPA1 are activated by cold stimuli and various irritant chemicals including pungent organosulfur compounds and noxious unsaturated aldehydes such as acrolein (Guimaraes and Jordt, 2007) and have been shown to cause notable neurogenic inflammation, hyperactivity and nociception (Trevisani et al., 2007b, Du et al., 2007b, Streng et al., 2008). In cultured neurones from wild type mice, acrolein triggers Ca^{2+} influx whereas no response is seen in neurons of TRPA1-deficient mice (Bautista et al., 2006).

Accordingly, the bladder inflammation, extravasation and oedema observed after CPO or IFO administration may be neurogenically mediated. Plasma extravasation after CPO administration was partially inhibited by an $\alpha 1$ -AR antagonist (Trevisani et al., 2007a). In addition, intravesical botulinum toxin A has been shown to improve symptoms of PBS patients (Pinto et al., 2013) presumably due to decreased neuropeptide release and nociception (Chuang et al., 2004). Furthermore, TRPA1 antagonists inhibited extravasation in the urinary bladder of rats treated with CPO (Geppetti et al., 2008). Therefore, it is possible that acrolein may directly activate TRPA1 receptors on sensory nerves contributing to neurogenic inflammation and enhanced nociception. However, TRPA1 channels can also be activated by molecules produced during oxidative stress and endogenous inflammation (Trevisani et al., 2007b, Streng et al., 2008) such as that occurring after CPO or IFO treatment. Accordingly, the evidence suggests that the symptoms of CPO or IFO induced cystitis may be at least partially due to neurogenic inflammation triggered directly or indirectly by the toxic metabolites of CPO or IFO.

Mast Cells

Mast cells are also important contributors to the development of an inflammatory response. Their activation results in the release of mediators that trigger inflammation and neuronal hyperexcitability (Theoharides et al., 2001). Several studies have demonstrated a close

relationship between mast cells and afferent nerves and it is thought that mast cells play an important role in neurogenic inflammation (Harvima et al., 2010, Keith et al., 1995, Yano et al., 1989). Mast cells appear to be involved in CPO and IFO induced inflammation. An increased number of mast cells have been reported after acute and chronic CPO administration in mice and rats (Golubeva et al., 2014, Juszczak et al., 2010, Sakthivel et al., 2008, Hu et al., 2003). However, most importantly, Deberry et al., (2014) reported an increase in the number of degranulated mast cells one day after CPO administration in mice. Mast cell degranulation has been demonstrated in response to neuropeptides released from afferent nerves (Yano et al., 1989, Saban et al., 2002), acrolein administration (Hochman et al., 2014) and muscarinic receptor stimulation (Spanos et al., 1996). Damaged urothelial cells also produce cytokines or growth factors that can stimulate proliferation and activation of mast cells (Theoharides et al., 2001). Increased numbers of mast cells have been reported in painful bladder syndrome (Theoharides et al., 2001). The release of proinflammatory or nociceptive molecules from mast cells is postulated to cause sensitisation of nerves and further release of transmitters and peptides which can continue to stimulate mast cells. It is this cycle of perpetual stimulation between nerves, mast cells and urothelial cells that is thought to contribute to the ongoing symptoms of PBS (Theoharides et al., 2001).

The results outlined in this thesis provide evidence for increased ACh and cytokine release from urothelial cells after acrolein or CAA treatment. Accordingly, it is possible that the inflammation and afferent sensitisation caused by CPO or IFO is due to an increased number of mast cells and increased activation either directly by toxic metabolites or indirectly by the release of neuropeptides or transmitters from nerves or urothelial cells.

Effects of CPO or IFO Induced Bladder Damage and Inflammation

Effects on the Urothelium

Normal urothelium has a low turnover rate and is unresponsive to epidermal growth factor (EGF) in urine because the superficial cells lack epidermal growth factor receptors (EGFRs) (Romih et al., 2001). However, the partially differentiated cells in the basal or intermediate urothelial layers do express EGFRs and when exposed during injury the urinary EGF can stimulate their proliferation giving the urothelium its regenerative ability (Romih et al., 2001, Varley and Southgate, 2008, Jost et al., 1989).

Previous studies have demonstrated that CPO treatment leads to an acute period of urothelial damage resulting in loss of cells (Auge et al., 2013, Juszczak et al., 2010). This thesis has also

reported similar results and has shown that acrolein and CAA are capable of causing damage to the urothelium. This damage exposes the partially differentiated urothelial cells and would lead to intense proliferation and urothelial hyperplasia (Boudes et al., 2011, Golubeva et al., 2014). A normal three layered urothelium is restored within 2-3 weeks (Romih et al., 2001). These various stages of urothelial damage and recovery have different implications on bladder function. As demonstrated in cultured human urothelial cells and porcine tissues the urothelial cells are capable of secreting various mediators that can influence the underlying smooth muscle and nerves. Treatment with acrolein or CAA was capable of causing enhanced release of various mediators from individual cells. During the early stages of damage fewer urothelial cells were present to release mediators and the overall mediator levels were mostly unchanged. However, the implications of these changes in the setting of a hyperplastic urothelium could mean much higher levels of the inflammatory and stimulatory mediators ATP, ACh and PGE₂ causing further urothelial dysfunction, changes in detrusor activity and afferent sensitisation and activation. It is unknown whether these effects remain long term in the restored urothelium and it would be interesting to determine whether urothelial mediator release remains high 2-3 weeks after CPO or IFO treatment.

Effects on Spontaneous Activity

CPO treatment has been shown to cause enhanced spontaneous activity in the bladder of rodents (Pan et al., 2012, Eser et al., 2012, Okinami et al., 2014). The results in this thesis did not show any effect of CPO, IFO or their toxic metabolites on spontaneous activity after acute treatment whereas, most of the previous studies have demonstrated enhanced activity several days after CPO treatment. Accordingly, this may be a long term effect of CPO or IFO induced inflammation in the bladder. In support of this theory, similar increases in spontaneous activity have also been reported in inflammatory conditions such as interstitial cystitis and lipopolysaccharide (LPS) induced inflammation (Ikeda et al., 2009, Tambaro et al., 2014).

Bladder strips from cats with feline interstitial cystitis had significantly more spontaneous Ca²⁺ transients in the urothelial/LP layer compared to controls and the transients subsequently moved into the detrusor (Ikeda et al., 2009). In mice, LPS induced bladder inflammation and enhanced the amplitude of spontaneous bladder contractions of isolated whole bladders (Tambaro et al., 2014). An anti-inflammatory cannabinoidergic compound was found to reduce the inflammation in the bladder and also reduced the amplitude of spontaneous contractions. Accordingly it appears that inflammation may affect the urothelium/LP or possibly the detrusor making it hyperactive and causing increased spontaneous activity. The lack of change in

spontaneous activity during the acute stage of cystitis observed in this thesis also supports that this may be an inflammation dependent change that occurs at a later stage of cystitis.

Effects on Afferent Nerves

CPO and IFO treatment causes irritation and inflammation of the bladder as well as altered sensations and pain and these two effects may be closely related. Chronic irritation or inflammation can cause various changes throughout the sensory pathway which may contribute to the symptoms experienced by patients after CPO or IFO treatment (Forrest et al., 2013, de Groat and Yoshimura, 2009) and **Figure 7.1** shows an overview of these mechanisms.

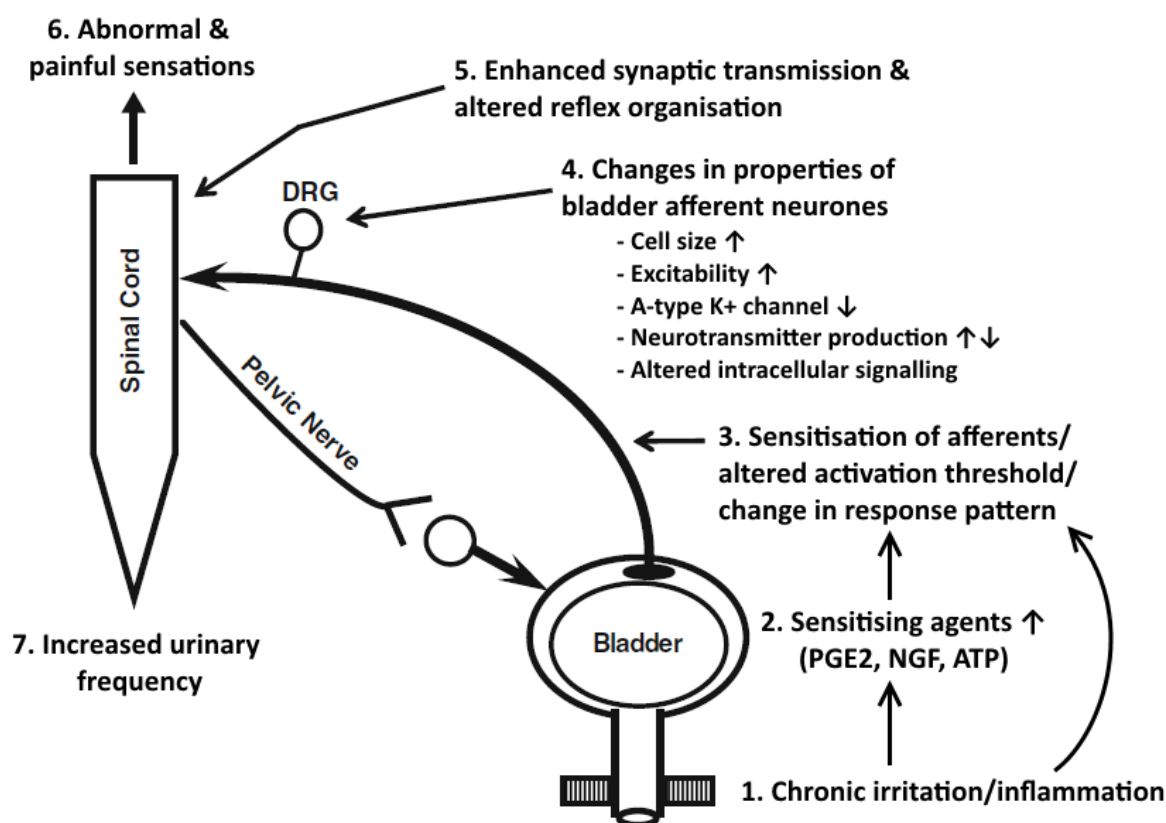


Figure 7.1: A summary of the events involved in chronic inflammation of the bladder and their contribution to hyperexcitability of the afferent neurons. The events that occur following chronic bladder inflammation (1) are indicated by sequential numbers (2–7). DRG dorsal root ganglia, PGE prostaglandin E, NGF nerve growth factor. Adapted from (de Groat and Yoshimura, 2009). Original author manuscript accessed via public domain through the Pubmed Central database.

Changes in the Properties, Transmission and Organisation of Afferent Nerves

Several studies have demonstrated increased immediate early gene expression (*c-fos*) in spinal neurones and altered expression patterns in regions of the lumbosacral spinal cord after CPO treatment (Vizzard, 2000, Dinis et al., 2004, Trevisani et al., 2007a, Lanteri-Minet et al., 1995). Expression of *c-fos* is thought to link short-term signals from extracellular stimuli to long term changes in gene expression and is considered a marker for increased neuronal activity (Sagar

et al., 1988). Furthermore, changes in *c-fos* expression may also be indicative of altered sensory pathway organisation and micturition reflexes. After CPO treatment of rats, CGRP and substance P immunoreactivity was significantly increased in fibres in L6-S1 spinal cord as well as in DRG involved in micturition reflexes and supports the idea that changes in neurotransmission may contribute to altered sensations during cystitis (Vizzard, 2001).

CPO treated rats have also demonstrated increased bladder afferent nerve firing (Yu and de Groat, 2008), while isolated DRG neurones demonstrated enhanced excitability to a current stimulus (Yoshimura and de Groat, 1999, Dang et al., 2008). This suggests that CPO treatment sensitises individual nerve fibres resulting in higher total nerve activity. The data in Chapter 4 supports this theory and indicates that sensitisation of individual low threshold fibres is responsible for the overall increase in activity. However, total nerve activity may be increased further as a result of a lower activation threshold in the high threshold fibres and altered response profiles of non-encoding low threshold fibres to an encoding response. The mechanism responsible for this afferent sensitisation is not yet clear.

Urothelial Release of Neuromodulatory Mediators

As discussed throughout this thesis, various mediators released by the urothelium can activate and sensitise afferent nerves such as ATP and PGE₂ (Rong et al., 2002, Ishizuka et al., 1995b, Su et al., 2008). Although initial results in cultured human urothelial cells indicated that release of both excitatory mediators was enhanced after acrolein or CAA treatment, the effects of systemic CPO and IFO treatment in mice did not show increased levels of either mediator. This may be due to the fact that metabolism of the parent drugs *in vivo* takes time and the mice bladders would not have been exposed to toxic metabolites for as long as the cultured cells. Similarly, the concentrations of acrolein and CAA in the urine *in vivo* may not have been as high as the concentrations applied to the urothelial cells. In any event, the increased sensory activity seen after CPO or IFO treatment in the mice does not appear to be mediated by ATP or PGE₂ from the urothelium. However, this does not rule out a role for these mediators in the later stage effects of CPO or IFO induced cystitis, but indicates that another mechanism of afferent sensitisation contributed to the acute effects seen in this thesis.

Another compound released by the urothelium during inflammation is NGF which is thought to alter pain signalling. Intravesical administration of NGF can induce nociceptive responses and bladder hyperactivity (Chuang et al., 2001, Dmitrieva et al., 1997). Urinary NGF concentrations have been shown to be increased after CPO treatment in mice (Boudes et al., 2011) and NGF-sequestering molecules have been shown to reduce the CPO induced bladder overactivity

in rats (Hu et al., 2005). NGF binds to tyrosine kinase receptors (Trks) and their expression and phosphorylation was enhanced in lumbosacral nerves of rats after CPO treatment (Qiao and Vizzard, 2002). The density of both parasympathetic and peptidergic sensory fibres was enhanced in the urothelium/lamina propria and detrusor of rats treated with CPO suggesting that an increase in the density of afferent and efferent nerves could contribute to the urinary symptoms experienced by patients treated with this drug (Dickson et al., 2006). Furthermore, the parasympathetic and sensory fibres were often found wrapped around each other with close association of the varicosities making it possible that interactions between these two fibre populations is altered by CPO induced cystitis. It is likely that increased NGF expression contributes to the increased density of nerves seen after CPO treatment. NGF can also cause up regulation of P2X receptor transcripts in afferent nerves which may sensitise them to ATP and increase bladder sensations in the inflamed bladder (Ramer et al., 2001).

Reactive Oxygen Species

ROS have been implicated in afferent nerve sensitisation in several organs and the mechanism of this could be via direct actions on the nerves or indirectly by stimulating production of neuromodulatory mediators. The reactive oxygen species hydrogen peroxide (H_2O_2) has been shown to activate TRPA1 receptors in mouse DRGs causing Ca^{2+} influx and non-selective cation currents (Sawada et al., 2008). As TRPA1 channels are expressed in some nociceptive sensory neurones this suggests that ROS may be able to directly alter afferent activity and pain sensations. Furthermore, ROS formation was found to cause sensitisation of rat lung vagal afferents to capsaicin and ATP (Shen et al., 2012). Application of H_2O_2 to rat larynx also resulted in enhanced reflex activity in this tissue (Tsai et al., 2007). However, the sensitisation of the laryngeal afferent nerves could be prevented by ATP scavengers or a P2X antagonist suggesting involvement of ROS induced ATP release activating neuronal P2X receptors. Intravesical application of H_2O_2 in rats induced bladder overactivity characterised by a reduction in the intercontraction interval (Masuda et al., 2008). Capsaicin prevented the change in intercontraction interval suggesting sensitisation of the afferents was responsible for the overactivity. The detrusor overactivity could also be blocked by hydroxyl radical scavengers and COX inhibitors suggesting that the sensitisation could be partly mediated by ROS induced prostaglandin synthesis. The data presented in Chapter 3 of the present study demonstrated that both acrolein and CAA are capable of causing ROS production in urothelial cells. Accordingly, it is possible that ROS production after CPO or IFO treatment may be directly sensitising bladder afferents or even contributing to sensitisation indirectly via ATP or PGE_2 production. However, given the findings in Chapters 5 and 6, the increased afferent activity after

CPO or IFO treatment was not accompanied by an increase in ATP or PGE₂ suggesting that ROS may be having a direct effect on bladder afferents sensitising them to mechanical stimulation during bladder filling.

Altered Receptor or Channel Activity

Chronic bladder inflammation can also alter the responses of chemosensitive receptors such as the TRP channels in sensory neurons. For example, isolated DRG neurons from cats with interstitial cystitis show larger responses to the TRPV1 agonist capsaicin and slower desensitisation compared to controls (Sculptoreanu et al., 2005). Furthermore, the altered TRPV1 receptor activity could be reversed by inhibiting protein kinase C suggesting that changes to the intracellular signalling pathway were responsible for the enhanced responses. Systemic CPO or intravesical acrolein treatment in wild type mice caused bladder hyperactivity and sensitisation of the paw withdrawal response to mechanical stimulation whereas these effects were not seen in TRPV1 knockout mice (Wang et al., 2008b). In mice, systemic IFO treatment resulted in visceral nociception and this could be inhibited by the TRPA1 antagonist HC-030031 suggesting a role for acrolein or inflammatory products in activating nerves (Pereira et al., 2013). Therefore, it appears that TRPV1 and TRPA1 receptors may be involved in the afferent hyperactivity due to CPO or IFO.

Capsaicin sensitive C-fibre afferents from rats treated with CPO exhibited lower thresholds for spike activation and demonstrated a tonic rather than phasic firing pattern (Yoshimura and de Groat, 1999). These neurones also had an increased somal diameter and suppressed A-type K⁺ channel currents. A reduced A-type K⁺ current could be a key contributor to the afferent hyperexcitability in CPO induced cystitis. No studies have looked at the changes to A δ -fibres after CPO or IFO treatment, however in cats with interstitial cystitis the A δ -fibres were more sensitive to pressure changes than in controls (Roppolo et al., 2005). This suggests that both C-fibres and A δ -fibres are likely to undergo functional changes in response to CPO or IFO induced cystitis.

Concluding Remarks

As with any avenues of scientific research, the work in this thesis has generated a number of questions and interesting avenues for future investigation. Firstly, the results presented here support a role for CAA and not just acrolein in the urotoxicity of CPO and IFO. Specifically, both acrolein and CAA were found to be toxic to human urothelial cells and caused damage to porcine bladder sections when applied luminally. Both acrolein and CAA induced urothelial cell death was associated with an increase in ROS production providing support for the theory that

oxidative stress is a major contributor to the urotoxicity of CPO and IFO. Furthermore, ROS production appeared to be linked to apoptotic death of urothelial cells in response to both acrolein and CAA.

Very little information is available about the urinary concentrations of these metabolites in a clinical setting. Accordingly, it would be advantageous to measure the actual concentrations of acrolein and CAA appearing in the urine of animals and humans treated with CPO or IFO. This would confirm the clinically relevant concentrations for future studies and would allow assessment of the significance of previous studies. Confirming whether CAA appears in the urine after CPO or IFO treatment and elucidating the concentrations of the metabolites would also have significant ramifications for correct dosing of protective agents.

The work in this thesis has provided support for the theory that CPO and IFO treatment both cause increased bladder afferent nerve activity due to an enhanced firing rate of individual low threshold nerve fibres (Yu and de Groat, 2008). It also suggests that total nerve activity may be increased further as a result of a lower activation threshold of high threshold fibres and/or an altered response profile of non-encoding low threshold fibres. Determining actual urinary concentrations of CAA and acrolein would also allow for intravesical application of these agents in the mouse model and further investigation of their effects on individual nerve responses. This would enable clarification of which metabolite is responsible for the afferent sensitisation.

As discussed in the previous Chapters, detrusor tone, spontaneous contractile activity and excitatory urothelial mediators can alter afferent nerve activity. However, the enhanced afferent nerve activity seen in this thesis was not associated with increased excitatory urothelial mediator release or altered detrusor tone. This suggests that CPO or IFO treatment is able to enhance nerve activity via a mechanism independent of bladder function and that the bladder pain and urinary hyperactivity experienced by patients is primarily due to sensitisation of the afferent pathways. It is my hypothesis that this may be due activation of TRP channels directly by the toxic metabolites acrolein or CAA or by a secondary effect due to mediators produced during inflammation and oxidative stress.

The antioxidant and GSH precursor NAC demonstrated promising protection of the human urothelial cells to acrolein and CAA induced damage. It would be interesting to determine whether oral supplementation of the mice before and/or during CPO or IFO treatment could prevent the changes in afferent nerve activity.

The bladder inflammation caused by CPO and IFO treatment appears to have a significant impact on the function of the bladder as well as the afferent nerve activity. Further research into how inflammation in this setting alters urothelial and sensory nerve contribution to overall bladder function could further our understanding of other overactive and inflammatory bladder conditions.

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